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NEGATIVE FEED-BACK CONTROL CAUSED BY THREONINE AND METHIONINE IN L-HOMOSERINE FERMENTATION

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In 1948, TEST *et al.* (*1*) found a mutant strain of *Neurospora crassa* which requires both methionine and threonine for its growth, and also recognized that homoserine was able to replace the roles of those two amino acids. From this evidence, they suggested that homoserine might be a precursor for those two amino acids. In 1951, FLING and HOROWITZ (*2*) also reported that a methionine-less mutant of *Neurospora crassa* accumulated some amount of homoserine and threonine in its mycelia. Since then, it has become clear that homoserine is an important intermediate in the biosynthesis of essential amino acids such as threonine (*2*, *3*), methionine (*2*, *3*), and isoleucine (*3*, *4*).

During the studies on fermentative production of various amino acids, the present authors found two threonine-less mutants of *Micrococcus glutamicus* (*5*) to have accumulated significant amounts of L-homoserine in their culture broths. This was reported in a preliminary paper (*6*), and the details of the fermentation were also reported in previous papers (*7*, *8*, *9*, *10*).

The present study confirmed the so-called "negative feed-back control" phenomena caused by threonine and methionine in the fermentative production of L-homoserine by *Micrococcus glutamicus* 534-Co 147 strain, which is a threonine-less mutant. In order to clarify the "negative feed-back control" phenomena, further experiments were carried out with both growing cultures and intact cell suspensions, and the possible sites of the "negative feed-back control" in the pathway of homoserine biosynthesis were discussed.

EXPERIMENTAL METHODS

(I) *Growing Culture Experiments*

(a) *Strains used*: Among many auxotrophs of *Micrococcus glutamicus* isolated by NAKAYAMA *et al* (*11*) and many other species, two strains, i. e., *M. glutamicus* 534-185 and 534-Co147 (threonine-less mutants) were found to show a considerable accumulation of L-homoserine in their culture broths (*7*).

In the present experiments, only *M. glutamicus* 534-Co147 strain was used.

(b) *Culture media and methods*: For seed culture, glucose-bouillon medium (glucose 1%, peptone 1%, meat extract 1% and sodium chloride

0.5%; pH 7.0) was used. One loopful of the organism was transferred from a stock culture slant (glucose-bouillon medium with 2% agar-agar) to 10 ml of seed medium in a sterilized test tube (2.3 cm dia.), and the test tube was incubated on a reciprocal test-tube shaker for 24 hours at 30°.

One ml of seed culture thus obtained was transferred into 25 ml of fermentation medium in a 250 ml-ERLENMYER'S flask, and the flask was shaken at 28° on a rotary shaker (220 r.p.m., 5 cm dia.).

Composition of fermentation media is given in each experiment described in the text.

Two parallel flasks were run for each analytical test in the growing culture experiments.

(II) *Intact Cell Experiments*

(a) *Preparation of intact cell suspension*: Ten ml of seed culture prepared as described in the preceding section was inoculated in 250 ml of fermentation medium in a 2 L-ERLENMYER'S flask with two buffles, and the flask was shaken on a rotary shaker at 30°. After 40 hours' culture, the fermentation broth was harvested and centrifuged for 5 minutes at 400 r.p.m. (30 cm dia.) in order to eliminate calcium carbonate from the broth. Then, the broth was again centrifuged for 20 minutes at 4,000 r.p.m. (30 cm dia.) in order to collect bacterial cells. The cells were then washed twice with sterilized 0.9% sodium chloride solution by centrifugation. The washed cells were resuspended in sterilized M/15 potassium phosphate buffer (pH 7.3). The optical density of the snspension was measured at 660 mμ, and the cell density was calculated from a preliminarily prepared calibration curve and then adjusted to 100 mg (dry cells)/ml with the same buffer.

(b) *Preparation of reaction mixture and incubation*: Reaction mixture contained 350 μM of potassium phosphate buffer (pH 7.35), 10 μM of magnesium sulfate, 100 μM of glucose, 200 μM of ammnium sulfate and 100 mg (in dry weight) of intact cells in total volume of 5 ml. The reaction mixture was placed in a test tube (1.5 cm dia.), and incubated on a reciprocal shaking device for 6 hours at 30°. After the incubation, the mixture was heated for several minutes in a boiling water bath in order stop the reaction, and then centrifuged. The clear supernatant thus obtained was used for homoserine and lysine assays.

As the blank, zero-time sample was treated in the same way, and the amounts of homoserine and lysine measured were corrected for those of the blank.

In order to get each analytical data, duplicate tube cultures run in parallel were assayed.

(III) *Analytical Methods*

(a) L-*Homoserine assay*: Quantitative determination of L-homoserine in culture broths and reaction mixtures was carried out by a turbidimetric bioassay method (*7*) with *Escherichia coli* 177-184 (*12*) which was kindly

furnished by Dr. B. D. DAVIS. L-Homoserine in culture broths and reaction mixtures was successfully determined by this method in the range between 10 to 50 μg/ml of L-homoserine.

(b) *Determination of lysine*: Lysine in culture broths and reaction mixtures was determined by a colorimetric method of NELSON (*13*).

(c) *Determination of glucose*: Glucose in culture broths was determined by a colorimetric method of SOMOGYI (*14*).

(d) *Measurement of bacterial growth*: One ml of fermentation broth was placed in a conical centrifuge-tube and centrifuged for 10 minutes at 3,000 r.p.m. (20 cm dia.), and then the supernatant was discarded. One ml of 2*N* hydrochloric acid was then added to the tube in order to dissolve calcium carbonate which contaminated in bacterial cells. Then the bacterial cell suspension in 2*N* hydrochloric acid was immediately made up to 20 ml with distilled water.

Optical density of the cell-suspension was measured by an electrophotometer with a 660 mμ filter, and used as a measure for bacterial growth.

RESULTS

(I) *Growing Culture Experiments with Chemically Defined Media*

(a) *Effect of biotin and L-threonine on the bacterial growth and the production of L-homoserine and L-lysine*

M. glutamicus has originally been a biotin requiring organism (*15*). And also, *M. glutamicus* 534-Co147 strain, the organism used here, has been found to be a threonine requiring mutant. Therefore, it is interesting to examine the effect of biotin and threonine on both bacterial growth and L-homoserine productoin.

In Table 1, amounts of L-homoserine produced and bacterial growth under various concentrations of biotin and of L-threonine are shown. Also, amounts of L-lysine produced are presented in the same table, because L-lysine has been found to be another main product in this fermentation (*6*, *7*).

As shown in Table 1, increased amount of biotin caused better growth of bacteria, while L-homoserine and L-lysine production was rather stable if biotin concentration in the medium was 5 μg/l or more. However, if the concentration of biotin was less than 5 μg/l, both bacterial growth and production of L-homoserine and L-lysine decreased to a great extent, and production of glutamic acid became predominant.

On the other hand, concentration of L-threonine caused more distinct change in the production of both amino acids. At 400 to 500 μg/ml of threonine concentration, usually 10 mg/ml or more of L-homoserine was accumulated in the culture media. Maximum production of L-homoserine was attained in a medium containing 400 μg/ml of L-threonine and 5 μg/l of biotin. In this case, 13.5 mg/l of L-homoserine and 9.7 mg/ml of L-lysine were produced.

The results of Table 1 were diagramatically shown in Figure 1.

Table 1. Effect of L-Threonine and Biotin on L-Homoserine and L-lysine Production in Growing Culture Experiments.

Ingredient added to the medium		Growth (O.D.)	pH	L-Homoserine produced (mg/ml)	L-Lysine produced (mg/ml)
L-Threonine μg/ml	Biotin μg/l				
80	0	0.025	5.6	0.30	0.60
	5	0.270	5.6	2.05	3.11
	10	0.271	6.1	2.22	4.08
	30	0.312	5.7	2.35	3.58
200	0	0.036	5.6	2.80	1.13
	5	0.470	6.2	3.05	5.66
	10	0.481	6.1	4.11	5.91
	30	0.573	5.5	4.39	7.68
300	0	0.041	5.7	0.75	1.38
	5	0.605	5.6	7.05	8.77
	10	0.655	5.6	8.15	12.40
	30	0.686	5.6	8.65	11.71
400	0	0.041	6.3	1.01	0.89
	5	0.600	5.6	13.50	9.70
	10	0.673	5.6	10.93	9.25
	30	0.681	5.6	12.63	8.80
500	0	0.058	5.6	1.52	1.15
	5	0.600	6.9	10.18	8.80
	10	0.710	6.5	11.75	7.81
	30	0.736	7.2	10.33	7.85
600	0	0.058	5.7	0.62	1.00
	5	0.690	8.6	8.23	6.35
	10	0.710	8.5	7.90	4.10
	30	0.735	8.7	6.85	4.01

Basal medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2%, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7aq$ 0.03%

Fermentation was run at 28° for 4 to 5 days.

From Figure 1, the response of homoserine and lysine production to the concentration of L-threonine can easily be seen. Productivity of lysine was predominant over productivity of homoserine if the concentration of L-threonine was less than 300 μg/ml. On the contrary, homoserine production became predominant over lysine production if the concentration of L-threonine was more than 300 μg/ml.

Based on the evidence indicated above, the effect of biotin and L-threonine on the fermentative production of amino acids by *M. glutamicus* 534-Co147 is schematically shown in Figure 2.

(b) *Inhibitory effect of excess amount of* L-*threonine on the production of* L-*homoserine and* L-*lysine*

As shown in Figure 1, highest production of homoserine was usually attained when L-threonine level in the medium was 400 to 500 μg/ml. If the concentration of threonine was increased to more than 500 μg/ml, a marked

decrease of homoserine production was observed. A similar tendency was also observed in the case of lysine production in which more than 300 μg/ml of threonine always caused a severe depression of lysine production. From these results, it was supposed that a higher concentration of threonine in the culture medium gave some inhibitory effects on both homoserine and lysine production.

In order to get clearer results on the inhibitory effect of threonine, growing culture tests with feeding various amounts of threonine were carried out. The results are shown in Table 2.

As shown in Table 2, addition of L-threonine did not cause any change in bacterial growth. However, both homoserine and lysine productions were decreased to a great extent. In this case, significant accumulation of lactic acid was observed paperchromatographically. Similar results were observed in glutamic acid fermentation (*21*) and valine fermentation (*22*). Glycine was also detected paperchromatogrophically in culture broth when excess threonine was supplied. This suggested degradation of excess threonine to glycine by threonine aldolase (*23*, *24*).

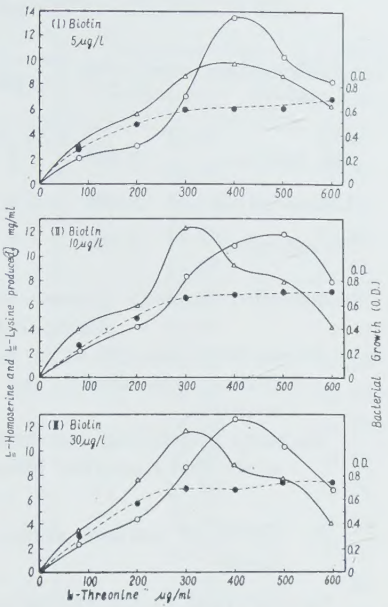

Fig. 1. Effect of L-Threonine and Biotin on Homoserine and Lysine Production.

○——○ Homoserine
△——△ Lysine
●- - -● Growth (O.D.)

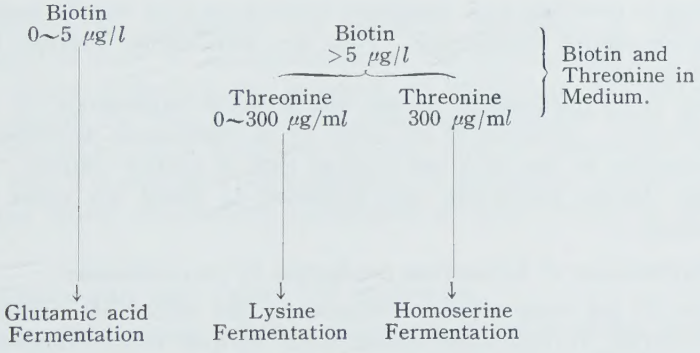

Fig. 2. Effect of Biotin and L-Threonine on Glutamic acid, Lysine, and Homoserine Production by *Micrococcus glutamicus* 534-Co147 Strain.

Table 2. Inhibition of Homoserine and Lysine Production by Threonine.

L-Threonine added	Growth (O.D.)	pH	L-Homoserine produced (mg/ml)	L-Lysine produced (mg/ml)	Lactic acid produced	Glycine produced
No addition	0.596	6.2	11.22	10.00	−	−
1.0 mg/ml×3	0.578	7.6	2.97	2.70	+++	+
2.0 mg/ml×3	0.590	7.5	3.14	2.60	+++	+

Fermentation medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7aq$ 0.03%, biotin 7.7 r/l and L-threonine 400 μg/ml.
Fermentation was run at 28° for 91 hours.
L-Threonine was added in the medium intermittently every 24 hours after inoculation.
Bacterial growth was measured at the 31st hour and the other data were obtained at the 91st hour after inoculation.

(c) *Effect of various amino acids on the production of homoserine and lysine*

As described in the preceding section, the inhibitory effect of L-threonine on the homoserine and lysine production has now become clear. In amino acid metablolism, some antagonism between different amino acids (*16, 17, 18*) and feed-back mechanisms (*19, 20*) have already been known. Therefore, effects of many amino acids other than threonine on this fermentation were also examined.

These amino acids were added to the starting medium individually and on the 5th day culture broths were analyzed for pH, bacterial growth, and for the production of both homoserine and lysine. The results obtained are shown in Table 3.

None of the amino acids used increased homoserine production. On the contrary, some of them were rather inhibitory on homoserine production. It is interesting to note that such inhibitory amino acids have usually been closely related to homoserine metabolism. They are methionine, cystine, homocysteine and so forth.

Among them, methionine caused the severest suppression of L-homoserine production. Addition of 200 μg/ml of DL-methionine decreased homoserine production to less than one-third of that of control culture. On the other hand, lysine production was increased to about 1.5 times that of control culture.

(d) *Suppression of homoserine production by* DL-*methionine*

In order to get more reliable evidence on the effect of methionine in this fermentation, further experiments with various concentrations of DL-methionine in the starting medium were carried out. Those results are shown in Table 4.

Table 3. Effect of Amino Acids on L-Homoserine Production.

Amino Acid Added	pH	Growth (O.D.)	L-Homoserine produced (mg/ml)	L-Lysine produced (mg/ml)
No addition	7.7	0.607 (+++)	14.65	12.00
D, L-Alanine	8.4	++±	13.95	10.60
L-Arginine·HCl	8.4	+++	13.03	14.00
L-Glutamate (Na)	8.3	+++	12.95	12.90
Glycine	8.1	+++	14.45	15.60
L-Histidine·HCl	8.4	+++	15.83	11.40
D, L-Isoleucine	8.4	+++	12.00	8.60
L-Leucine	8.4	+++	11.33	10.00
L-Lysine·HCl	8.4	+++	14.28	15.40
D, L-Methionine	5.8	+++	4.00	17.70
D, L-Phenylalanine	7.7	+++	15.03	10.90
L-Proline	8.2	+++	12.40	13.00
D, L-Serine	8.1	+++	11.48	12.00
D, L-Tryptophan	8.4	+++	12.10	9.80
L-Tyrosine	8.3	+++	11.93	7.60
L-Valine	8.3	+++	13.13	8.50
L-Aspartate (Na)	8.3	+++	9.45	9.20
L-Cystine	5.8	0.680	9.93	9.70
D, L-Homocysteine	5.9	0.601	7.28	10.10
D, L-Homoserine	8.4	+++	10.63	11.20
D, L-α-aminobutylic acid	7.4	+++	9.70	6.80

Fermentation medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2%, L-threonine 400 μg/ml, biotin 6.5 μg/l, K_2HPO_4 0.1% and $MgSO_4$·7aq 0.03%; Each amino acid was added in the medium in an amount of 200 μg/ml.

Fermentation was run at 28° for 5 days.

Homoserine production was markedly decreased by the addition of 200 μg/ml or more of DL-methionine in the starting medium. On the contrary, lysine production was increased by addition of methionine. Interestingly enough the total amounts of both homoserine and lysine produced were rather constant (about 20 mg/ml) at every level of methionine concentration. From this, it is presumed that suppression of homoserine production by methionine has forced intermediary metabolism to move forward to lysine production.

(II) *Experiments with Intact Cell Suspension*

From the growing culture experiments described above, it has become evident that threonine and methionine have inhibitory effects on homoserine production. In order to observe the mode of those inhibitory effects in

Table 4. Inhibition of L-Homoserine Production by D, L-Methionine.

Concentration of D, L-methionine $\mu g/ml$	Growth (O.D.) at 3rd day	4th day			5th day		
		pH	Homo-serine mg/ml	Lysine mg/ml	pH	Homo-serine mg/ml	Lysine mg/ml
0	0.890	5.6	13.08	7.89	7.1	14.38	9.58
50	0.910	5.6	12.38	9.48	7.1	13.25	9.53
100	0.845	5.8	10.58	9.83	6.8	11.33	15.25
200	0.845	5.8	4.35	13.10	6.0	5.75	16.15
300	0.765	6.1	3.23	13.70	7.2	3.40	17.55
400	0.770	7.2	4.70	11.65	7.3	3.90	14.22
600	0.825	7.1	4.90	14.65	8.1	3.50	14.90
800	0.815	7.1	5.18	15.15	8.3	4.80	14.75
1,200	0.805	7.2	6.75	14.75	8.3	5.70	14.45

Fermentation medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2%, L-threonine 400 $\mu g/ml$, biotin 7.5 $\mu g/l$, K_2HPO_4 0.1% and $MgSO_4 \cdot 7aq$ 0.03%.
Fermentation was run at 28°.

broader light, experiments with intact cell suspensions were carried out.

(a) *Effect of methionine on the formation of enzyme system for homoserine and lysine biosynthesis*

M. glutamicus 534-Co147 strain was grown on three different media, i. e., (1) synthetic medium, (2) synthetic medium containing DL-methionine and (3) natural medium. The compositions of these media are shown in Table 5. Bacterial cells were harvested from each culture broth and intact cell suspensions were prepared as described in the part on experimental methods. The results obtained from the experiments with these intact cell suspensions are shown in Table 5.

As shown in Table 5, homoserine productivity was predominant over lysine productivity in the cells obtained from the synthetic medium. On the other hand, cells from the methionine-containing synthetic medium showed much decreased homoserine productivity and much increased lysine productivity. Cells from the natural medium also showed decreased homoserine productivity but the lysine productivity was not so different as that of cells obtained from synthetic medium.

These results were quite coincident with those of growing culture experiments (see Table 4), and this supported the presumption that methionine might repress formation of some enzyme system for homoserine biosynthesis.

(b) *Effect of* DL-*methionine on the enzyme activity for homoserine and lysine biosynthesis*

In the preceding section, the effects of DL-methionine on the formation of enzyme systems for homoserine and lysine biosynthesis were examined. Further investigations on the effect of DL-methionine on the enzyme activity

Table 5. Homoserine and Lysine Synthesizing Abilities of Intact Cells Obtained from Different Media.

Cells Harvested	L-Homoserine synthesized	L-Lysine synthesized
From synthetic medium	18.7 μM	12.5 μM
From synthetic medium +D, L-methionine	10.1 μM	23.4 μM
From natural medium	12.1 μM	12.8 μM

Synthetic medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2%, L-threonine 400 μg/m, biotin 6.5 μg/l, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7aq$ 0.03%.

Synthetic medium+D, L-methionine: 400 μg/ml of D, L-methionine was added to the above medium.

Natural medium: glucose 10%, $(NH_4)_2SO_4$ 2%, $CaCO_3$ 2%, NZ amine 1.0%, yeast extract 0.5%, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7aq$ 0.03%.

Composition of Reaction Mixture: Potassium phosphate buffer (pH 7.3) 350 μM, $MgSO_4 \cdot 7aq$ 10 μM, glucose 100 μM, $(NH_4)_2SO_4$ 200 μM, and intact cells 100 mg (dry wt.) in 5 ml of total volume.

Reaction mixture was incubated at 30° for 6 hours on a shaking device.

for homoserine and lysine biosynthesis were carried out. Cells from synthetic medium and methionine-containing medium (see Table 5) were also used. Different amounts of DL-methionine as indicated in Table 6 were added in the reaction mixtures, and the amounts of homoserine and lysine synthesized were assayed. The results are shown in Table 6.

Neither enzyme activity for homoserine nor lysine biosynthesis was inhibited by DL-methionine.

Table 6. Effect of D, L-Methionine on Homoserine and Lysine Syntheses by Intact Cells.

Cells Harvested	Concn. of D, L-methionine in reaction mixture**	Homoserine synthesized	Lysine synthesized
From Synthetic medium*	None	18.5 μM	12.5 μM
	10^{-4} M	22.4 〃	13.1 〃
	10^{-3} 〃	20.8 〃	15.5 〃
	10^{-2} 〃	22.0 〃	19.4 〃
From Synthetic medium +D, L-methionine*	None	10.1 〃	23.4 〃
	10^{-4} M	9.4 〃	20.9 〃
	10^{-3} 〃	10.5 〃	21.4 〃
	10^{-2} 〃	11.6 〃	30.5 〃

* Compositions of fermentation media used were the same as indicated in Table 5.

** Compositions of reaction mixtures were the same as indicated in Table 5. D, L-methionine was added in the reaction mixture in an amount as described above. Reaction mixture was incubated at 30° for 6 hours on a shaking device.

Summarizing the results presented in Tables 5 and 6, it can be said that DL-methionine represses the formation of certain enzyme system for homoserine biosynthesis but does not inhibit the enzyme activity for the same.

(c) *Effect of* L-*threonine on the enzyme activity for homoserine and lysine biosynthesis*

NAKAYAMA *et al.* (*20*) reported that both threonine and homoserine strongly inhibited the enzyme activity for lysine production in the lysine fermentation with a homoserine requirring mutant of *M. glutamicus*. As were shown in Figure 1 and Table 2, excess amount of threonine strongly inhibited the production of homoserine. Therefore, the effect of L-threonine on the enzyme activity for homoserine and lysine production was investigated with intact cells from both synthetic and methionine-containing media. In Table 7, the experimental results are shown.

Table 7. Effect of L-Threonine on Homoserine and Lysine Synthesis by Intact Cells.

Cells harvested	Concn. of D, L-methionine in reaction mixture**	Homoserine synthesized	Lysine synthesized
From synthetic medium*	None	18.7 μM	12.5 μM
	10^{-4} M	20.8 〃	8.5 〃
	5×10^{-4} M	3.8 〃	1.7 〃
	10^{-3} M	4.6 〃	0.1 〃
	5×10^{-3} M	8.8 〃	0.0 〃
From synthetic medium +D, L-methionine*	None	10.1 〃	23.4 〃
	10^{-4} M	8.4 〃	29.1 〃
	5×10^{-4} M	7.4 〃	14.5 〃
	10^{-3} M	5.8 〃	3.8 〃
	5×10^{-3} M	4.8 〃	0.0 〃

* Compositions of fermentation media used were the same as indicated in Table 5.

** Composition of reaction mixture was the same as indicated in Table 5. L-Threonine was added to the reaction mixture in an amount as described above. Reaction mixture was incubated at 30° for 6 hours on a shaking device.

From Table 7, it can be seen that the addition of more than 5×10^{-4} M of L-threonine in the reaction mixture has caused a severe decrease in both homoserine and lysine productions. From this, it is evident that L-threonine strongly inhibited the enzyme activities for both homoserine and lysine biosyntheses.

DISCUSSION

Based on the experimental results described above, the metabolic pathway for homoserine biosynthesis and the possible sites of "negative feed-back control" mechanisms caused by threonine and methionine are schematically shown in Figure 3.

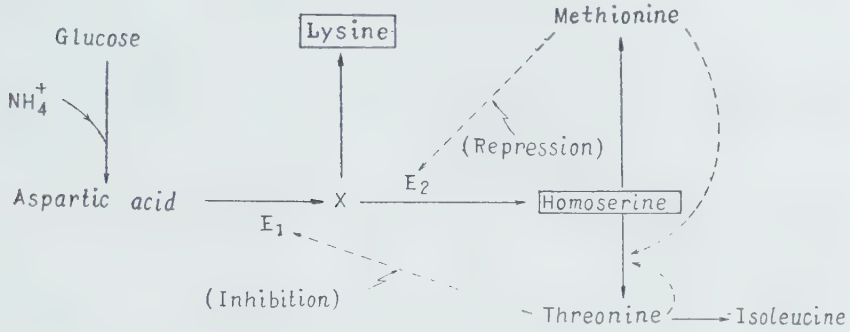

Fig. 3. Schematic Diagram for the Biosynthesis of Homoserine and Lysine, and Possible Sites of "Negative Feed-Back Control" Mechanisms Caused by Threonine and Methionine.

——→ Biosynthetic Path. - - -→ Inhibition (or Repression)

(a) *M. glutamicus* 534-Co147 strain is a threonine requiring mutant and can accumulate a large quantity of L-homoserine in the culture broths if it is cultivated in appropriate media. From this evidence, it can easily be presumed that a certain enzyme system between homoserine and threonine might be blocked. According to the reports by DAVIS (*25*), and BLACK and WRIGHT (*26*), it was suggested that both homoserine and lysine were synthesized from aspartic acid via a common intermediate X (see Figure 3). In the fermentation with *M. glutamicus* 534-Co147 strain, both homoserine and lysine were extracellularly accumulated in large amounts. This may give an additional support to the correctness of the assumption mentioned above.

(b) The present authors wish to discuss on the two "negative feed-back control" mechanisms caused by threonine and methionine in this fermentation. As previously described by PARDEE (*27*), there are two types of "negative feed-back control" mechanisms. One is "repression" which is suppression of enzyme formation, and the other is "inhibition" which is suppression of enzyme activity.

In the present fermentation, methionine suppressed the formation of certain enzyme system for homoserine biosynthesis (see Table 5). So, it is "repression." And also, in this case, the decrease of homoserine production was always accompanied by an increase of lysine production. Therefore, the "repression" by an methionine might occur on certain enzymes at a stage between an intermediate "X" and homoserine. If the enzyme system is designated as "E_2," E_2 must be repressed by methionine. Repression of E_2 by methionine may decrease the conversion of X to homoserine and, as the result, the intermediate X may be forced to move forward to lysine production. This assumption can reasonably explain the experimental results.

On the other hand, threonine suppressed the enzyme activities for both homoserine and lysine biosyntheses. So, it is "inhibition." Simultaneous inhibition of the production of both amino acids suggested that this inhibition

might occur on a certain enzyme or enzymes prior to the X formation. If the enzyme system is designated as "E_1", E_1 must be inhibited by threonine (see Figure 3).

MAGASANIK (*27*) has commented on the "negative feed-back control" mechanism as follows: "In general, only the first enzyme of a particular biosynthetic sequence seems to be subject to inhibition by the ultimate product, while many of the enzymes of the sequence may be subjected to repression." According to his comment, the assumption presented in Figure 3 must be considered reasonable.

(c) Concerning the pathway for threonine biosynthesis from homoserine, WORMSER and PARDEE (*28*) reported the inhibition of homoserinekinase by threonine, and FUJII *et al.* (*29*) reported that methionine inhibited the conversion of homoserine to threonine in growing culture experiments. Together with the two "negative feed-back control" mechanisms found by the present authors, therefore, at least four types of inhibition or repression lie on the biosynthetic pathway of threonine from glucose. This can account for the fact that threonine was scarcely found in culture broths when the microorganisms were cultivated in conventional media.

(d) In the recent advance in fermentative production of amino acids, auxotrophic mutants of *M. glutamicus* have played significant roles, for example, biotin-less strains in glutamic acid fermentation (*21*), homoserine-less strains in lysine fermentation (*20*), arginine-less strains in ornithine fermentation (*30*) and so forth. In order to achieve good yields of products in those fermentations, appropriate amounts of required substances were always necessary. However, it is common throughout these fermentations that an excess amount of substances required was always injurious to the production of amino acid wanted, though it stimulated bacterial growth more strongly. Such phenomena have also been found in the homoserine fermentation with *M. glutamicus* 534-Co147 strain. The strain requires threonine for the homoserine production as well as for its growth. However, an excess amount of threonine caused a sharp decrease of homoserine production, while bacterial growth was rather stimulated.

These facts may have important meaning not only in the amino acid fermentations using auxotrophic mutants but also in many other fermentative productions with auxotrophs.

SUMMARY

Fermentative production of L-homoserine was achieved by using a threonine requiring mutant of *Micrococcus glutamicus*. This strain produced a large quantity of L-homoserine as well as L-lysine in the culture broths if it was cultivated under appropriate conditions.

During the studies on fermentative production of L-homoserine, it was found that L-threonine concentration affected the formation of L-homoserine and L-lysine to a great extent. Maximum productions of L-homoserine and

L-lysine were attained when L-threonine concentrations were 400μg/ml and 300 μg/ml, respectively. And an excess amount of threonine caused a sharp decrease in both amino acid productions. The effects of various amino acids on this fermentation were also examined. As the results, two "negative feed-back control" phenomena caused by threonine and methionine were observed.

Further investigations using intact cell suspension revealed the mode of action of the two amino acids in the "negative feed-back" mechanisms. Experimental results showed that threonine inhibited a certain enzyme activity involved in the biosynthetic path of homoserine, and methionine repressed the formation of a certain enzyme on the same path.

Based on these experimental results the sites of these "negative feed-back" mechanisms were discussed.

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STUDIES ON MICROBIOLOGICAL TRANSFORMATION OF STEROID COMPOUNDS

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Since PETERSON and MURRAY (*1*) showed in 1952 that progesterone could be enzymically converted into 11α-hydroxyprogesterone by a fungus, *Rhizopus nigricans*, numerous investigations on microbial transformation of steriods have been carried out intensively.

These studies have made it increasingly clear that the ability to transform the steroid molecule is widespread among various kinds of microorganisms and the types of reaction encountered are numerous. The authors, also, have been studying on this subject since 1956. In our experiments, the screening test on various microorganisms such as fungi, bacteria and streptomycetes of about 700 strains for steroid transforming ability was carried out by paper chromatography, and several strains which were found to have a comparatively strong ability to transform steroids were selected in different types of reaction. These strains were then applied to various kinds of corticoid steroids such as progesterone, deoxycorticosterone, REICHSTEIN's compound S, corticosterone, cortisone, or hydrocortisone and the steroids transformed were isolated and their structures determined. It was found that almost all of these microorganisms investigated showed a marked substrate specificity in their steroid transforming reactions.

The results are described below.

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EXPERIMENTALS

I. *Examination of Steroid Transforming Ability of Microorganisms on Paper Chromatography*

First, to test the ability to transform a steroid, these microorganisms

were incubated in 20 ml. of medium* in a 50 ml. volume L-type flask at 26–28° for 1–2 days on a reciprocal shaker and 0.4 ml. of methanol solution of 10 mg. of REICHSTEIN's compound S or progesterone was added to each flask. The incubation was further carried on for 2–3 days under the same conditions. At the end of this period, the transformed steroid was extracted from the whole broth by shaking with 20 ml. of ethyl acetate and the extract was concentrated to dryness *in vacuo*. The residue was dissolved in a minimal amount of methanol and was spotted on a filter paper. For paper chromatography, a modification of ZAFFARIONI's method (*2*) was used, that is, a mixture of propylene glycol and methanol (1:2) as the stationary solution and a mixture of toluene and dioxane (78:22) as the developing solvent were used in the descending method. The detection of a steriod spot on the paper was made by ultraviolet absorption for a Δ^4-3-ketone or by appearance of pink color with triphenyltetrazolium chloride reagent solution.(*2*)

The results obtained are listed in Table 1.

Table 1. Position of spots on paperchromatography of steroidal transformation products formed by various microorganisms.

A. Fungi

Microorganism	Standard Oxygenated Steroids: 11α-OH	6β-OH	11β-OH	21-OH	Δ^1-3-keto**
(*a*) *Hypochnus centrifugum*	+				
Glomerella lagenarium					
Gloeosporium kaki	++				
Corticium vagium	+				
Ophiobolus miyabeanus				+	
Pircularia oryzae	++				
Gloeosporium laeticola	+				
Gibberella saubinetii		+			
Fusarium lycopersici		+			
Sclerotinia libertiana			++		
(*b*) *Glomerella gosypii*					
Diaporthe numurai					
〃 〃 (*A-47*)			+		
Hypochnus sasaki	+				
Corticium sp. (*B-57*)					
〃 〃 (*B-64*)					
Colletotrichum sp. (*F-38*)					
〃 *lindemuthiamum*					
Absidia regnieri	++		±		
Corticium sp. (*B-60*)					
〃 *vagum*	++				
Sclerotinia libertiana (*A-4*)				++	
(*c*) *Cercospora oryzae*					
〃 *kikuchii*					
Corticium graminum					
Colletotrichum phomoides	++				
Ceratostolla finbriata					
Fusarium lini	+				
Sclerotium hydrophilum	++				
Ophiobolus graminis	++				
Helicobasidium mompa					
(*d*) *Fusarium niveus*		++			
Glomerella lagenarium	++				
Botrytis cinerea				+	
Endotinia parasitica					
Sclerotinia allii					
Gloeosporium olivarum	++				

* In this experiment, the media used for fungi consisted of 5% glucose, 2% peptone and 0.3% corn steep liquor, or potato decoction containing 3% glucose; the media used for bacteria and streptomycetes, consisted of 1% glucose, 1% peptone, 1% meat extract and 0.3% sodium chloride (pH 7.0).

** In this Table, the presence of a spot of the substrate steroid was not indicated.

Microorganism \ Standard Oxygenated Steroids	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	**	s
Rosellinia necatrix							
Coniothirium Diplodiella	‡						
Glomerella fluctigena	‡						
Elsinoe ampelina							
Cercospora lagenarum							
Cladosporium fulvam (*S–10–1*)							
Fusarium lycopelsici							
Sphaceloma sp.							
Hypochnus centrifugus							
Botrytis favae (*Pi-1*)			+				
Alternaria kikutiana							
Sclerotinia fluctigena							
Venturia pirma							
Gloeosporium laeticolar	‡						
Taphrina diformans							
〃 *pruni*							
Phomopsis citri							
Alternaria citri							
Colletrichum gloeosprioides	‡						
Phytomonus citri							
Oospora aurantii							
Phytophthora citrophthora (*Ci–16*)							
Botrytis cinerea (*CI–17*)		‡	+				
Sclerotinia libertiana			‡	+	+		
Elsinoae fawcetti							
Mycosphaerella Horii							
Pestalozyia funerea							
Phytomonas Eicobatryae							
Gloeosporium kaki	‡						
Fusicladium diospiri (*B–1*)							
Botorytis cinerea		‡					
(*e*) *Alternaria bataticola*					+		
Cerotostomella fimbriate							
Gloeosporium kaki (*438*)							
Gibberella fujikuroi							
Ascophyta pinadella							
Ervinia aroideae							
Pleurotus ostreatus							
Piricularia oryzae		‡					
Ascochyta favae							
Cortinellus shiitake	+						
Phytophthora parasitica							
Helicobasidum mompa							
Corticium roefsii							
Polystictus cinnabarius							
Corticium salmonicolar							
Cochliobolus Niyabeanus							
Xanthomonas citri					+		
(*f*) *Rhizoctonia solani* (*B–5*)							
〃 〃 (*P–20*)							
〃 *sp.*					‡		
Sclerotinia sclerotiorum			‡	+			
Phoma sp.							

Microorganism \ Standard Oxygenated Steroids	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	**	s
Fusarium lini							
Cerocospora baticola							
Hypochnus sasaki							
Corticium centrifugus							
(*b*) *Irpex consors*							
Polyporus frondosus							
Polystictus hirstus							
〃 *polyzonus*	+	+					
〃 *sanguin·ns*							
Shizophyllum commune							
Frametes pini							
Pleurotus japonicus							
Stereum induratum							
Echinodontum tsugicola (*IAM 2–2*)							
〃 (*IAM 2–3*)							
Fomes robstus							
〃 *sp.*							
Lenitzites betulina	+	+					
〃 *tennis*							
〃 *styracina*							
Polyporus sulphureus							
〃 *pubescens*	+	+					
Polystictus versicola	‡						
Trometes dickinsii							
Lenitzites sepiaria							
Irepex lacteus	+	+					
(*c*) *Helminthosporium sigmoideum*							
〃 *irregulare*							
〃 *zonatum*							
Ophiobolus graminis							
〃 *sativus*		+					
(*g*) *Pyrenophora teres* (*A–17*)							
〃 (*A–29*)	+	‡					
Helm. zonatum (*A–30*)							
〃 *sp.* (*B–8*)							
〃 *avenae*							
〃 *turcicum*							
〃 *siccuns* (*B–15*)							
〃 *sp.* (*B–36*)						‡	
〃 *sp.* (*B–37*)						‡	
〃 *turcicum* (*B–34*)							
Ophiobolus heterostropus						‡	
Helm. turcicum (*B–51*)							
〃 *sp.* (*B–66*)	‡						
〃 *sp.* (*B–69*)							
〃 *sp.* (*B–70*)							
〃 *sp.* (*B–74*)	‡						
〃 *turcicum* (*F 1–73*)							
〃 *sativam* (*F 1–40*)							‡
(*h*) *Helm. oryzae*							

Standard Oxygenated Steroids / Microorganism	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	**	S
〃 *lepochloae*	+						
〃 *kusanoi*		+					
〃 *maydis*							
〃 *coices*	+	+					
〃 *setariae*	+						
〃 *avenae*							
〃 *tritici-vulgaris*		+					
〃 *leersii*	+	+					
〃 *sativum*		+					
〃 *gramineum*							
〃 *brizoe*							
(a) *Helm. turcicum* (4068)							
〃 *nodulosum*							
(i) *Pythium ultimum*		+					
Circinella sydowi		+					
Mucor mucedo	+	+					
Parastiela simplex							
Rhizopus nigricans	+	‡					
Cunninghamella echinulata	‡	+					
Mortiella pusilla		+					
Dipodascus albidus							
Byssochlamys nivea							
Endomyces lindneri							
Pichia rosa							
Aspergillus condidus							
〃 *flavus*							
〃 *niger*							
Penicillium citrinum							
〃 *luteum*							
Elsinae ampelina							
Pyrenophora greminea			+				
Allescheria boidii							
Carpenteles javanicus	+						
Chaetomium globosum	‡			‡			
Neurospora sitophila							
Diaporthe nomurai							
Glomerella cingulata	‡						
Daldinia concentria	+	+					
Xlyaria polymorpha	‡						
Cochliobolus miyabeanus	+	+					
Ophiobolus miyabeanus	+						
Cordyceps militaris	+	‡					
Gibberella saubinetti	‡	+					
Guignardia camelliae	‡						
Pyronema conbluens	‡						
Ascochyte vicial							
Diplodia natalensis	‡						
Phomopsis citri	+						
Pestalotia diospyri	‡						
Geotrichum lactis							
Sporobolomyces roseum							
Tilletiopsis lilacina							
Acremonium potronii							
Acrostalagums albus	+	‡					

Standard Oxygenated Steroids / Microorganism	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	**	S
Beauveria bassiana	‡						
Botrytis reptons	‡	+					
Candelospora penicilloides	‡						
Cladosarum olivaceus	‡						
Corethropsis hominis							
Epidermophyton floccasum							
Gliocladium roseum							
Hyalopus nopporoensis	+	+					
Monosporium apiospermum							
Mycogene jaepii	+						
Paecilomyces varitoi							
Piricularia oryzae	‡	‡					
Sarcinomyces crustaceum							
Scopulariopsis americana							
Spicaria viridans	‡		+				
Sporotricum asteroides							
Sterigmatocystis japonica							
Tricophyton concentricum	‡	‡	+				
Tricothecium roseum							
Tritiachium pururеum							
Verticillum nieveostratosum							
Alternaria citri							
Cerosporina kikuchii							
Cerospora oryzae	‡						
Chalara mycoderma							
Cladosporium herbarum							
Curvularia tetramera							
Pullularia pullulans							
Fumago sp.							
Helicocreas oryzae							
Helm. avenae							
Hemispora rogosa							
Hormodendrum pedrosoi							
Humicola grisea							
Macrosporium bataticola							+
Margarinomyces sp.							
Nigrospora orgzae	+	+					
Phialophora verrucosa							
Stachylidium bicolar	+		+				
Stemphylium botryosum							
Thielavia basicola		+					
Thyrospora astragali							+
Torulopsis aeria							
Isaria farinosa	+	‡	‡				
Stysanus medius	‡	‡					
Epicoccum purpurascens							
Fusarium oxysporum							
Histoplasma capsulatum							
Parendomyces asteroides							
Pseudomycoderma miso							
Corticium centrifugum	+	‡		‡			
Tomentella sp.							
Clavaria mucida	‡	+					
Irpex lacteus	+						
Fomes pinicola							
Ganoderma applam	+						

Microorganism	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	3-keto
Lenzites styracina						
Polystictus sanguineus	++					
Poria vaporaria						
〃 *sp.*						
Agaricus capentris						
Collybia velutipes						
Lepiota procera						
Pholiata adiposa						
Pleurotus ostratus						
(j) *Hurodake (15)*	+-	+-				
Kawaradake (16)		+-				
Aragekawaradake (20)	±	+-				
Shiyudake (24)	+-					
Kaigaradake (27)	+-	+				
Enokidake (27)	+-					
Polystictus versicolorpus	+-	+				

a) Strains preserved at Sankyo Co., Ltd.
b) 〃 〃 Inst. of Appl. Microbiology, Univ. of Tokyo
c) 〃 〃 Nat. Agri. Res. Inst.
d) 〃 〃 Tokai-Kinki Nat. Agri. Exp. St.
e) 〃 〃 Fac. of Agri., Kyushu Univ.
f) 〃 〃 Fac. of Agri., Hokkaido Univ. and Hokkaido Pref. Agri. Exp. St.
g) 〃 〃 Nat. Inst. of Health of Japan.
h) 〃 〃 Nagawo Inst.
i) 〃 〃 Fermentation Res. Inst., Agency of Ind. Sci. & Tech.
j) Strains distributed from Mr. Morimoto of Sankyo Co., Ltd.

Table 1, B. Bacteria***

Microorganism	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	3-keto
Ps. aureofaciens NRRL B-1543				++		
Ps. cruciviae P-8-2				± ++		
Ps. dacunhae A-6-3				++		
〃 *A-7-2*				+ ++		
〃 *R-10-2*				+ ++		
〃 *G-1-7*				++		
〃 *G-1-13*				++		
Ps. ovalis S-5				++		
〃 *A-10-3*				++		
〃 *C B-3*				++		
Ps. schuylkill NRRL B-6				++		
CB-6	+			++		
Ps. riboflavinus				++		
Ps. gelidicola				++		
Ps. fluorescens IFO 3081				++		
〃 *IFO 3459*				++		
〃 *Stanier A-3-8*				++		
〃 *A-3-12*				± ++		
Ps. sp. Kral				++		
Ps. jaegeri AHH-27				++		
〃 *AHH-23*				++		
〃 *AHH-28*				++		
M. lysodeikticus				++		
M. luteus 19-2				++		
M. ureae S-4				++		
M. variaus P-9-3				++		
Micrococcus sp.				++		
St. aureus FAD 209P		±		++		
S. albiden	±			++		
S. aurentiace	±			++		
S. lutea PCI 1001				++		
S. marginata				++		
S. variabilis	±			++		
Ach. cyclocoastes AN-17	±			++		
Alc. faecalis ACC 101				++		
〃 *ACC 107*				++		
E. coli coli-2				++		
〃 *coli-6*				++		
〃 *coli-7*		+		++		
〃 *coli-8*	±	±		++		
〃 *ML-3*		±		++		
〃 *Bordet*				++		

*** All strains were preserved at the Inst. of Appl. Microbiology, Univ. of Tokyo.

Standard Oxygenated Steroids / Microorganism	11α-OH	6β-OH	11β-OH	2β-OH	Δ¹	S
〃 *Najiar*						++
〃 *ATCC 3635*						++
A. aerogenes Izaki						++
〃 *ATCC 7256*						++
〃 *ATCC 8308*						++
〃 *ATCC 8724*						++
〃 *ATCC 8329*						++
A. cloacae A 1-2						++
〃 *1-1*						++
〃 *K-5*					±	++
S. marcescens 1-2					±	++
〃 *1-3*					±	++
〃 *1-7*					±	++
〃 *1-9*					±	++
〃 *1-10*				±		++
〃 *2-1*						++
〃 *2-2*						++
〃 *2-3*				±		++
〃 *2-5*					±	++
〃 *2-7*					±	++
〃 *2-10*	±				±	++
〃 *IFO 3048*						++
〃 *IFO 3046*						++
B. subtilis NRRL B-558					±	++
〃 *PCI 219*					±	++
〃 *IFO 3026*				±		++
〃 *IFO 3036*	+			±		++
〃 *IFO 3035*						++
〃 *IFO 3033*	+				±	++
〃 *IFO 3027*						++
〃 *ACTU B-3-3*				±		++
〃 *ACTU B-3-4*				±		++
〃 *ACTU B-3 5*				±		++
〃 *ACTU B-5-6*				±		++
〃 *PCI 220*				±		++
〃 *Hay-1*	+			±		++
〃 *Hay-2*				±		++
〃 *Hay-3*				±		++
〃 *Hay-4*				±		++
〃 *Hay-5*				±		++
〃 *Hay-6*				±		++
〃 *Hay-8*	+			±		++
〃 *Natto 1-2*						++
〃 *Natto 1-3*						++
〃 *Natto 1-4*				±		++
〃 *Natto 1-5*					±	++
〃 *Natto 1-6*						++
〃 *Natto 1-7*						++
〃 *Natto 1-8*					±	++
〃 *Natto 1-9*	+					++
〃 *Natto 1-10*	±					++
〃 *Natto II-1*						++
B. cereus Murao	±	++	++		+	++
〃 *IFO 3001*	±					++
〃 *IFO 3131*					±	++
〃 *DC-3*	±					++
〃 *IFO 3039*						++
〃 *IFO 3015*	+					++
〃	±	+	+		±	++
B. megaterium 6-1			±	±		++
〃 *899 (1)*	+					++
〃 *37-1*						++
〃 *EC-34*				±		++
〃 *12-1*	±	+				−
B. polyomyxa NRRL B-694						++
〃 *IFO 3020*						++
B. sphericus						++
B. pumilus						++
B. alvei IFO 3343	±					++
B. brevis IFO 3331						++
B. circulans IFO 3329						++
〃 *IFO 3342*						++
B. firmus IFO 3330						++
B. macerans IFO 3483						++
〃 *IFO 3490*						++
B. roseus IFO 3041					±	++
Agro. tumefaciens IFO 3058						++
Cory. equi						++
Cory. fascians						++
Cory. hoagi						++
V. metschnikovu						++
V. tyrogenes Shin-Denke						++
Ps. aeruginosa MT						++
〃 *Baltimore*						++
Pro. vulgaris HX-19					±	++
B. thiaminolyticus						++
B. coagnlans ATCC 7050						++
〃 *P-22*						++
〃 *P-33*						++
〃 *P-55*						++

Table 1. C. Streptomycetes

Microorganism****	Transformation
Streptomyces sp. No. 8, 27, 44	16α-Hydroxylation on progesterone
Streptomyces sp. No. 41, 103	6β-, and 11α-Hydroxylation on progesterone

**** The examination was done with about 200 strains of unidentified streptomycetes isolated from soil. In this table, only the strains which were found to have an ability to transform progesterone on a paper chromatogram were described.

The sign (+) shows only the existence of the transformation product whose Rf value was close to that of the standard steroid and not the existence of the standard itself.

Symbols, 11α-OH, 6β-OH, 11β-OH, 2β-OH, Δ^1 and S for the standard oxygenated steroids indicate 11α-, 6β-, 11β-, 2β-hydroxy compound S, 1-dehydro compound S and compound S, respectively.

From Table 1, it appears that the ability to bring about changes in the steroid molecule is very widespread especially in fungi in contrast with the ability in bacteria or in streptomycetes, and in addition fungi show more varieties in types of steroid transforming reactions.

II. *Transformation of Various Steroids by the Selected Microorganisms*

From the results of screening test in Part I, several microorganisms which had a strong steroid transforming ability, showing respective types of reaction on steriods were selected.

These microorganisms were then further tested on each of various corticoid steroids and the transformed steriods were isolated and their structures were determined. In these experiments, about sixty kinds of transformed steroids were isolated, among which eleven steroids were new compounds.

In these experiments, the strain of microorganisms was grown in ten to one hundred flasks of 500 ml. volume each containing 100 ml. of the medium above described on a reciprocal shaker for 1-2 days at 26-30° and to each flask 50 mg. of the requisite substrate steroid dissolved in 2 ml. of methanol was added, and the fermentation was continued for the additional 2-3 days under the same conditions. The culture was then filtered and the transformed steroids were extracted with ethyl acetate followed by washing with sodium bicarbonate and with water. The ethyl acetate extracts dried over anhydrous sodium sulfate were evaporated to dryness *in vacuo* and the residue was either recrystallized directly from a solvent like acetone or methanol, or separated by adsorption column chromatography. The determination of the structure of transformed steroid isolated was performed by chemical treatments and physical measurements, etc. The results obtained are listed in Table 2 together with corresponding references.

Table 2. Transformation products formed by selected microorganisms from various steroids.

Class	Micro-organism \ Substrate	Progesterone	17α-Hydroxyprogesterone	Deoxycorticosterone	Reichstein's comp. S	Corticosterone	Cortisone	Hydrocortisone	Ref.
Fungi	*Sclerotinia libertiana*	**2β, 15β-OH**	**2β-OH** 11α-OH (15β-OH)	**2β, 15β-OH**	2β-OH (11α-OH) (11β-OH)	**2β-OH 15β-OH**			1
	Absidia regnieri	14α-OH 6β, 14α-OH **9α, 14α-OH 7α, 14α, 15β-OH**	11α-OH	14α-OH	11α-OH	**14α-OH, 11-CO**			2
	Botrytis cinerea		6β-OH	6β-OH (11β-OH)	6β-OH (11β-OH)	**15β-OH, 11-CO** 15β-OH			3
	Glomerella lagenarium	11α-OH 6β, 11α-OH	11α-OH	11α-OH	11α-OH	(−)			4
	Gloeosporium kaki	〃	〃	〃	〃	〃			4
	Sclerotium hydrophilum	6β, 11α-OH	6β-OH 11α-OH	**6β, 11α-OH**	6β-OH 11α-OH	6β-OH, 11-CO 15β-OH, 11-CO			5
	Stachylidium[a] *bicolar*			14α-OH 11β-OH	11β-OH	(−)			6
	Fusarium lycopersici	15α-OH	6β-OH	15α-OH	6β-OH	15, x-OH			7
	Gibberella saubinetti	〃	〃	〃	〃	〃			7
	Gliocladium roseum *Helminthosporium turcicum* *Ophiobolus heterostropus*			Δ^1	Δ^1	Δ^1	Δ^1	(−)	8
	Alternaria bataticola	5β-H_2 3α-OH 3β-OH	5β-H_2 3α-OH 3β-OH	5β-H_2 3α-OH 3β-OH	5β-H_2 3α-OH 3β-OH	5β-H_2 3α-OH	5β-H_2 3α-OH	5β-H_2 3α-OH	9
	Pythium[b] *ultimum*	Testololactone	Testololactone	Testololactone	20β-OH	(−)	(−)	(−)	10
Bacteria	*Bacillus sp. B-(A)*			Δ^1	Δ^1	Δ^1	Δ^1	Δ^1	11
	Pseudomonas[c] *dacunhae*			Δ^1 (decomp.)	Δ^1 (decomp.)	(Δ^1)	(−)	(−)	11
	Bacillus cereus	6β, 14α-OH		6β-OH 11α-OH 14α-OH	6β-OH 11α-OH	14α-OH, 11-CO			12
Streptomycetes	*Streptomyces sp. AC-27*	16α-OH	(−)	(−)	(−)	(−)			13
	Streptomyces sp. AC-103	6β-OH 6β, 11α-OH		6β-OH 11α-OH	6β-OH 11α-OH				13

-OH: Hydroxylated derivatives. 5β-H_2: 5β-Reduced product.
Δ^1: 1-Dehydro derivatives. ( ): In minimum amount.
11-CO: 11-Dehydro (11-keto) derivatives. Boldface: New steroid. (−): No reaction.

a. This microorganism oxidized 14α, 21-dihydroxy-4-pregnene-3,20-dione to give 11β, 14α,-21-trihydroxy-4-pregnene-3,20-dione.

b. This microorganism transformed androstendione into testololactone.

c. In the case of this microorganism deoxycorticosterone and REICHSTEN's compound S were subjected to 1-dehydrogenation as well as simultaneous degradation.

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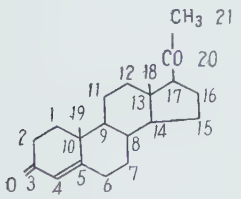
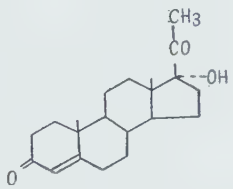
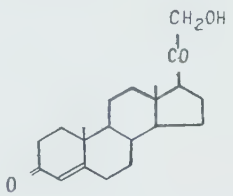
The transformed steroids obtained by cultivation of the selected microorganisms with various substrates are summarized in Table 2.



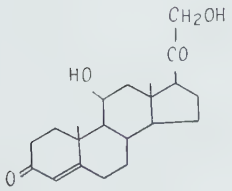
Progesterone



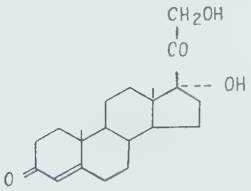
17α-Hydroxyprogesterone



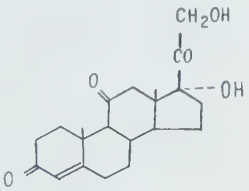
Deoxycorticosterone



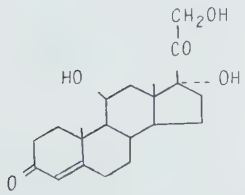
Corticosterone



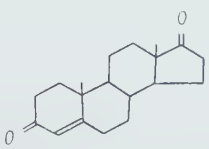
Reichstein's compound S



Cortisone



Hydrocortisone



Androstendione

RESULTS AND DISCUSSIONS

A. The results obtained in this experiment showed that almost every strain investigated possessed a certain type of remarkable substrate specificity in its steroid transforming reaction. Based on the types of transforming reactions the following classification may be considered.

1. Hydroxylation:

(i) Effect of the presence of a hydroxyl group in the substrate steroid on reaction—From the results shown in Table 2, it is assumed that 14α-hydroxylation by *Absidia regnieri*, *Bacillus cereus* and *Stachylidium bicolar*; 15α-hydroxylation by *Gibberella saubinetti* and *Fusarium lycopersii* and 15β-hydroxylation by *Sclerotinia libertiana* and *Botrytis cinerea* are inhibited by the presence of a 17α-hydroxyl group in the substrate steroid molecule. It is also assumed that 2β-hydroxylation by *Sclerotinia libertiana* and 6β-hydroxylatoin by *Botrytis cinerea*, *Sclerotium hydrophilum*, *Gibberella saubinetti*, *Fusarium lycopersici* and *Bacillus cereus* are inhibited by the presence of a 11β-hydroxl group in the substrate steroid molecule. In these cases, some kind of steric hindrance is considered to be one of the reasons.

(ii) Different specificities of reactions in 11β- and 14α-hydroxylation by *Stachylidium bicolar*—This fungus possesses both abilities to introduce hydroxyl groups at positions of 11β and 14α of the steroid. However, this fungus effected 11β-hydroxylation of the 14α-hydroxylated compound of deoxycorticosterone, but it failed to effect 14α-hydroxylation of the 11β-hydroxylated deoxycorticosterone (corticosterone). Consequently, there seem to be different kinds of substrate specificity between these two reactions caused by this fungus. This distinct difference in specificity of reaction seems to be due to the difference in permeability of the cell of this fungus against these two substrate steroid molecules.

(iii) Difference in patterns of reaction of 6β-and 11α-hydroxylation between fungi and streptomycetes.—In 6β-hydroxylation of progesterone it was found that there was a remarkable difference in bioconversion patterns between fungi and streptomycetes. That is, although both streptomycetes and fungi, for example, *Glomerella*, *Gloeosporium* in our experiments, or *Rhizopus* in PETERSON's (*1*) experiments produced the same 6β, 11α-dihydroxyprogesterone as a dihydroxylated product from progesterone, they produced different monohydroxyprogesterones; streptomycetes produced 6β-hydroxyprogesterone, whereas, the above-mentioned fungi invariably produced 11α-hydroxyprogesterone.

Probably the enzyme systems of these two kinds of microorganisms are different.

2. Dehydrogenation:

(i) Different specificities in 1-dehydrogenation of steroids among fungi, *Bacillus* sp. B-(A) and *Pseudomonas dacunhae*—In 1-dehydrogenation of steroids, it was observed that these three kinds of microorganisms had

different substrate specificities in this reaction, i.e. fungi effected 1-dehydrogenation of the steroids except hydrocortisone. *Ps. dacunhae*, also effected this reaction of steroids except the steroids having a 11-hydroxyl group. In contrast to this, in the case of *B.* sp. B-(A), this bacterium evenly effected 1-dehydrogenation of all steroids used, showing no substrate specificity in this reaction. It is assumed that the difference of these substrate specificities in the same 1-dehydrogenation by these microorganisms is due to a difference in permeability of the cell of each microorganism against each of substrate steroid molecule.

(ii) Substrate specificity in 11-hydroxy dehydrogenation—*Absidia regnieri* was found to effect dehydrogenation of 11β-hydroxyl group of corticosterone, but not of 11α-hydroxyl group, the epimer, so that this fungus has a kind of stereochemical specificity in this reaction.

3. Reduction:

(i) Substrate specificity in reduction of Δ^4 and 3-ketone of steroids by *Alternaria bataticola*—This fungus was found to reduce both the double bond and the 3-ketone of pregnene compounds to 5β-pregnane evenly and to 3-hydroxypregnane analoges. In the case of reduction of a 3-ketone, it was observed that this fungus reduced the steroid having no oxygen function at C-11 to both 3α-and 3β-hydroxy pregnane derivatives, but those having an oxygen at C-11 were reduced only to an isomer, 3α-hydroxy pregnane. So it appears that an oxygen at C-11 of the steroid influences the stereochemistry of hydrogenation of pregnane to 3-hydroxy.

(ii) Substrate specificity in degradation of the side chain and in reduction of the 20-ketone by *Pythium ultimum*—*Pythium ultimum* was found to effect degradation of the side chain and D-ring of the steroids having only one hydroxyl group or none in the side chain into testololactone equally, and in contrast, it effect reduction of a 20-ketone to a 20β-hydroxy derivative without any degradation in the case of steroids having two hydroxyl groups in the side chain like dihydroxyacetone side chain.

B. In these experiments, the following eleven new steroid compounds were obtained:

(i) 2β, 15β, 21-Trihydroxy-4-pregnene-3, 20-dione
(ii) 2β, 11β, 21- 〃 〃
(iii) 6β, 11α, 21- 〃 〃
(iv) 7α, 14α, 15β- 〃 〃
(v) 11β, 14α, 21- 〃 〃
(vi) 11β, 15β, 21- 〃 〃
(vii) 2β, 15β-Dihydroxy 〃 〃
(viii) 2β, 17α- 〃 〃
(ix) 7α, 14α- 〃 〃
(x) 14α, 21-Dihydroxy-4-pregnene-3, 11, 20-trione
(xi) 5β, 21- 〃 〃

SUMMARY

Transformation of REICHSTEIN's compound S or progesterone by various microorganisms such as fungi, bacteria and streptomycetes of about 700 strains has been studied (Table 1). The actions of several strains upon various corticoid steroids were investigated and the transformed steroids were isolated and their structures determined. From these results it was revealed that almost all of the microorganisms tested have a certain kind of substrate specificity in their actions as shown in Table 2.

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CHANGE OF PANTOTHENIC ACID CONTENT IN *CHLORELLA* CELLS DURING THE SULFUR STARVATION AND SUBSEQUENT RESTORATION, AS STUDIED BY THE TECHNIQUE OF SYNCHRONOUS CULTURE

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INTRODUCTION

It has been shown in our preceding paper (*1*) that an abnormally large amount of fat was accumulated in *Chlorella* cells when they were grown synchronously in a sulfur-deficient medium and thus made incapable of performing cell division. The accumulated fat disappeared when the cell division was induced by incubating the S-starved cells in the medium containing sulfate plus nitrate under photosynthesizing conditions.

In the present work, attention was focused on the behavior of free and combined pantothenic acid during the process of S-starvation and subsequent restoration. Pantothenic acid is known to be—in combination with β-mer-

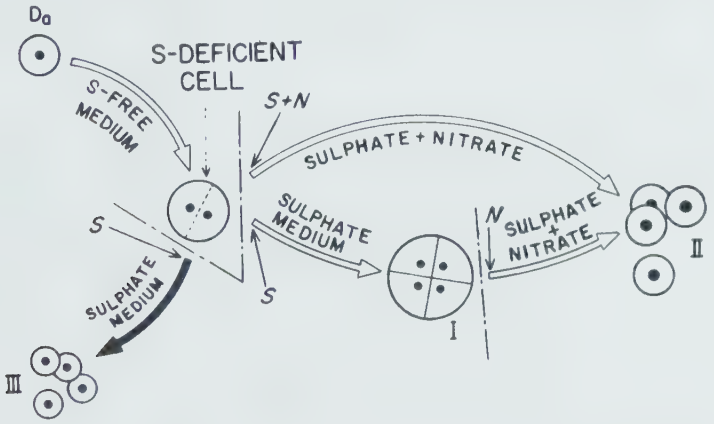

Fig. 1. Schematic representation of the course of life cycle of *Chlorella* as controlled by the limited supply of sulfur and nitrogen sources under photosynthesizing and non-photosynthesizing conditions. The white arrows indicate the processes under photosynthesizing conditions and the black ones the processes under non-photosynthesizing conditions (under illumination, but without provision of CO_2). For further explanations see text and also our previous paper (*2*).

captoethylamine and an adenosine derivative—a constituent of coenzyme A, which is an important factor not only in the fat metabolism but also in many other biochemical reactions.

It was found that the content of the combined pantothenic acid (presumably CoA) was low in the cells which had been made incapable of division by S-starvation, while it was strikingly high in the cells which were produced by division of the cells that were recovered from the S-starvation by either sulfate supplied alone under non-photosynthesizing conditions or sulfate plus nitrate under photosynthesizing conditions (Fig. 1). In view of these results and considering the fact that CoA is a sulfur-containing substance, it may be deduced that one of the essential roles of sulfur in the process of cell division of *Chlorella* may be that by virtue of some biocatalytic functions of CoA.

MATERIALS AND METHODS

(I) *Algal material*

The algal strain used was *Chlorella ellipsoidea*. The processes of S-starvation and the subsequent restoration under different conditions are illustrated schematically in Fig. 1. The algal cells used for analyses were obtained by the same ways as those described in the preceding paper (*1*). The cells at Stage III were obtained by incubating the S-deficient cells for 66 hours in a medium containing potassium sulfate alone under illumination but without provision of CO_2 (the suspension was aerated with CO_2-free air).

(II) *Method of analysis of pantothenic acid*

Immediately after harvesting, the suspension of algal cells was cooled with ice and centrifuged. The cells were washed twice with cold water. Extraction of pantothenic acid was made by boiling the washed cells in water for 5 min. After boiling, the suspension of cells was rapidly cooled and centrifuged. The residue was washed with a small amount of cold water. This extraction procedure was repeated again, which was found to be sufficient for virtually separating the free and combined pantothenic acid from algal cells. The supernatants and washings thus obtained were combined and divided into two portions, each of which was lyophilized and used for analysis.

(i) *Analysis of free pantothenic acid*

One portion of the extract was used for the bioassay of free pantothenic acid. *Lactobacillus arabinosus* was precultured at 30° for about 20 hours in a medium containing, per 100 ml: 1.0 g trypton, 0.5 g K_2HPO_4, 0.2 g glucose and 0.2 g yeast extract; pH 6.8. The bacterium thus precultured was centrifuged and washed twice with 0.85% NaCl under aseptic conditions and used for the assay. The assay medium consisted of 1.0 ml of the basal

medium* and 1.0 ml of algal extract. To the medium was added one drop of the bacterial suspension, and the incubation lasted for 72 hours at 30°. The acid produced was titrated with $N/10$ NaOH.

(ii) *Analysis of combined pantothenic acid*

The other portion of the algal extract was digested enzymatically by incubating a mixture of 1.6 ml of water solution of the lyophilized extract, 0.1 ml of peptidase,** 0.1 ml of phosphatase,*** and 0.2 ml of tris buffer (1 M, pH 8.0) at 37° for 3 hours. The digested sample was boiled and analysed for free pantothenic acid in the same way as described above. The control was run under the same conditions as those in the experiment by using water in place of the algal extract. The amount of combined pantothenic acid was estimated by subtracting the amount of free pantothenic acid from the total amount in the enzyme-digested sample.

RESULTS AND DISCUSSIONS

In Table 1 are reproduced the results of analyses for free and combined pantothenic acid and lipid in the algal cells at different stages of cell development (Fig. 1). From the data in the table the following facts emerge:

(i) In the normal cells, free pantothenic acid was accumulated at the stage of ripening (L-stage) and presumably was transformed into the combined form at a later stage of cell ripening or during the phase of division.

(ii) During the period of S-starvation, not only the formation of free pantothenic acid was markedly suppressed, but the formation of combined pantothenic acid was totally halted. An accumulation of a large amount of surplus lipid occurred during this period.

(iii) When the S-deficient cells were provided with sulfate alone under photosynthesizing conditions, the free pantothenic acid increased up to the level in normal L-cells, but there occurred no increase in the amount of the combined form.**** During the process the cells remained unable to perform division, and the surplus lipid content of the cells remained unchanged.

* The basal medium contained, per liter: casamino acids ("Nissan"), 10 g; L-cystine, 400 mg; DL-tryptophan, 200 mg; adenine sulfate, 20 mg; guanine hydrochloride, 20 mg; uracil, 20 mg; vitamin B_1, 200μg; vitamin B_2, 400μg; biotin, 0.8μg; p-amino benzoic acid, 200 μg; nicotinic acid, 400 μg; vitamin B_6, 800 μg; KH_2PO_4, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 400 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $MnSO_4 \cdot 4H_2O$, 20 mg; sodium acetate (anhydrous), 20 g; glucose 40 g.

** The homogenate of pigeon liver-acetone powder (Sigma) in ice-cold 0.02 M $KHCO_3$-solution (10%) was centrifuged at 2,000 r.p.m. for 20 min. The supernatant thus obtained was passed through Dowex-I resin to remove CoA, and subjected to successive centrifugations at 5,000 r.p.m. (1.5 hr) and at 30,000 r.p.m. (30 min). The supernatant obtained was used for the digestion.

*** Alkaline phosphatase (Sigma) was dissolved in cold water (about 0.7%).

**** This conclusion can be made by taking into consideration the result that the total cell dry weight increased during the process in question.

Table 1. Contents of Pantothenic Acid and Lipid in *Chlorella* Cells as They Were Affected by the Control of S-N-Supply.

	Per gram of dried algal material			
	Pantothenic acid			Lipid
	free	combined	total	
Normal D-cells	1.25 μg	8.7 μg	9.95 μg	—
Normal L-cells	4.5	7.5	12.0	—
S-deficient cells	1.4	4.5	5.9	2250 μmole
Cells at Stage I	3.6	2.5	6.1	2270
Cells at Stage II	4.2	11.5	15.7	500
Cells at Stage III	1.7	15	16.7	961

(iv) On providing the S-deficient cells with sulfate alone under non-photosynthesizing conditions or with sulfate plus nitrate under photosynthesizing conditions, the cells were rendered able to divide, and during this course of events the content in cells of combined pantothenic acid increased strikingly and the surplus lipid disappeared.

These results strongly point to an important role played by the combined pantothenic acid (presumably CoA) in the cell division of *Chlorella*.* Considering the accumulation and disappearance of surplus lipid (fat) occurring concomitantly with the lowering and rising, respectively, of the content of combined pantothenic acid in algal cells, it may be inferred that the combined pantothenic acid participates, supposedly in the form of acetyl CoA, in the energy yielding system operating in the process of cell division of the alga. In fact, it has been pointed out by TAMIYA et al. (*4*) that the respiratory energy is essentially needed for the algal cell division.

The role of sulfur played in the process of cell division seems to have multifarious aspects. HASE et al. (*5*, *6*, *7*) have shown the importance in the algal cell division of certain sulfur substances which were found in appreciable amounts in the hot acid-soluble fraction of dividing cells. In the S-starved cells, the formation of these substances—which have been found to be certain S-containing deoxypentosepolynucleotides (SDN)—was markedly affected according to whether the cells were supplied with sulfate under photosynthesizing or non-photosynthesizing conditions. A remarkable event occurring under photosynthesizing conditions was that a large amount of sulfur was incorporated into protein, and only a little went into SDN. No doubt, both protein and SDN require nitrogen sources for their formation. Under non-photosynthesizing conditions, the nitrogen required for the synthesis of SDN would be taken from some endogenous N-source(s), while

* Morimura has reported (*3*) that pantothenic acid was formed mostly during the ripening phase in the normal life cycle of *Chlorella*. This is in accordance with the present results.

under photosynthesizing conditions this source would be preferentially used for the protein synthesis, making it unavailable for the formation of SDN. Thus, the fact that the S-starved *Chlorella* cells behave differently under photosynthesizing and non-photosynthesizing conditions in their nutritional requirement for their performance of cellular division was explained as being due to the occurrence of a competitive relation between the processes of photosynthetic growth and cell division (HASE et al., *2*, *7*). The present study has brought to light the significance of combined pantothenic acid (CoA) in the process of algal cell division, and the experimental results indicate that a competitive relation similar to that described above also obtains in the formation of the factor (CoA) containing pantothenic acid as an essential component.

SUMMARY

1. During the processes of cell division of *Chlorella* as controlled by the limited supply of sulfur and nitrogen sources, the fate of free and combined pantothenic acid was investigated in connection with the accumulation and disappearance of surplus lipid investigated in the preceding work.

2. When the young and small algal cells (D-cells) were grown synchronously in an S-free medium, the cells grew to some extent, without, however, being able to perform cell division. In the cells thus rendered S-deficient, the formation of pantothenic acid in both free and combined forms was profoundly suppressed, whereas the lipid content increased to an abnormally high level.

3. When the S-starved cells were provided with sulfate alone under photosynthesizing conditions, the cells still remained unable to divide. During this period no significant increase in the amount of combined pantothenic acid occurred, although its free form increased up to the normal level. In this case, the accumulated lipid did not disappear.

4. When the S-starved cells were made capable of performing cell division on being provided with sulfate under non-photosynthesizing conditions, or with sulfate plus nitrate under photosynthesizing conditions, the combined pantothenic acid increased remarkably and the surplus lipid disappeared.

5. Discussions were made on the possible role of the combined pantothenic acid in the algal cell division and on the competitive relation between the processes of photosynthetic growth and cell division in respect to the formation of the factor (probably CoA) containing pantothenic acid as an essential component.

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STUDIES ON MUTANTS OF *BACILLUS SUBTILIS* REQUIRING D-GLUTAMIC ACID
I. ISOLATION OF MUTANTS

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Although synthetic pathways of the L-series of amino acids have been studied extensively by the use of biochemical mutants of microorganisms such as *Neurospora crassa* and *Escherichia coli* (*1*), there appears to be no published account of the synthesis of the D-series of amino acids using similar techniques. Meanwhile, D-glutamic acid and D-alanine have been described as essential components of bacterial cell wall (*2–7*), capsule (*8–10*), and peptide in spore (*11*). Furthermore, several antibiotics including bacitracin and gramicidin are known to comprise D-amino acids as the constituents (*12 13*).

The present author attempted, in this connection, to produce mutants requiring D-amino acids for growth and study their physiological significance in microorganisms. A strain of *Bacillus subtilis* was exposed to X-rays, and five mutants dependent on D-glutamic acid were isolated.

MATERIALS

Strain: A wild strain of *Bacillus subtilis* K was used as the starting material.

Media: The following three media were employed. MM medium comprises NH_4Cl 5 g., NH_4NO_3 1 g., Na_2SO_4 2 g., $MgSO_4 \cdot 7H_2O$ 0.1 g., $CaCl_2$ 1 mg., K_2HPO_4 3 g., KH_2PO_4 1 g., trace element solution 1 ml. in distilled water 1,000 ml. PH is 7.0 after sterilization. Glucose is added to the liquid and solid media in concentrations of 0.1% and 0.5%, respectively. The trace element solution consists of $ZnSO_4 \cdot 7H_2O$ 8,800 mg., $FeCl_3 \cdot 6H_2O$ 970 mg., $CuSO_4 \cdot 5H_2O$ 393 mg., $MnCl_2 \cdot 4H_2O$ 72 mg., $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 37 mg., $Na_2B_4O_7 \cdot 10H_2O$ 88 mg. and distilled water 1,000 ml. DG medium contains the components of minimal medium (MM) and 100 mg D-glutamic acid. CD medium comprises 0.1% casein hydrolysate and 100 mg D-glutamic acid in addition to the components of MM medium.

Chemicals: The amino acids used in this experiment were produced at the Ajinomoto Co., Inc., and recrystallized when necessary.

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EXPERIMENTS AND RESULTS

1. *Induction of mutation and isolation of mutants*

Spores were harvested from a 10-day culture of *B. subtilis* K on BURKHOLDER's nutrient agar (*14*), and a suspension containing 10^6–10^7 spores in 2 ml sterile saline was exposed to X-rays (Matsuda X-rays projector, 168 KV, 25 mA). The intensity at a distance of 18 cm was 1,000 roentgens per minute, and 20% of the spores survived at a dosage of 144,000 roentgens. The survivals were transferred to MM medium and treated with penicillin (75 units/ml) for 18 hrs. Plating was made onto DG medium after appropriate dilution. The replica plating method (*15*) devised by LEDERBERG and LEDERBERG was applied to the colonies appeared on the plate using MM and DG media. The type of colonies which grew on DG medium but not on MM medium were selected. The yield was only four out of about ten thousand colonies tested. These mutants will be described hereafter as 1a, 2a, 3d and 4a. Mutant 3a is a spontaneous mutant of 3d capable of growing in CD medium, in which the parental 3d cannot grow.

2. *Genetic stability of the mutants*

To test genetic stability of the mutants, vegetative cells harvested from a culture at the late logarithmic growth phase in DG medium were washed with 0.1 M phosphate buffer (pH 7.2), and, after appropriate dilution, plated on MM medium. The colonies that appeared on the plate after three days were considered revertants. The ratio to the total cells plated is to be recorded as "frequency of revertant."

As seen in Table 1, mutants 3a and 4a were considerably stable, while mutants 1a and 2a reverted at higher frequencies. Mutant 3d gave rise to prototrophic revertants in a frequency of 3×10^{-6}. Along with this, the mutation toward 3a-type was tested by plating cells of 3d on CD medium. The frequency was found to be less than 10^{-8}.

Table 1. Frequencies of revertants in five mutants

	Mutant strains				
	1a	2a	3a	4a	3d
No. of cells plated (a)	9.6×10^6	7.7×10^6	1.6×10^8	1.8×10^8	1.1×10^8
No. of colonies appeared on MM plate (b)	183	270	0	0	348
CD plate (c)	—	—	—	—	0
Frequencies of revertants					
MM÷(b/a)	2×10^{-5}	3.5×10^{-5}	$<10^{-8}$	$<10^{-8}$	3×10^{-6}
CD÷(c/a)	—	—	—	—	$<10^{-8}$

3. *Growth response to other amino acids*

By substituting D-glutamic acid in DG medium with other amino acids (D, L, or DL form, each 100 μg/ml), growth of mutants in these media were tested. The test was performed in two ways. In case liquid media were used, about 10^7 cells were inoculated into 10 ml medium in a MONOD tube. Cultivation was carried out on a seesaw-type shake machine at 37°, and growth was observed after 20 hours. In case solid media were used, about 10^8 cells were spread on MM medium, and five to eight paper discs were placed on each plate at a distance of 15 mm. A small quantity of one amino acid solution was given to one paper disc, and the plates were placed overnight at 37°. The presence of growth zone around a disc was judged to be a positive growth.

Results are shown in Table 2. From the table, the five mutants are classified into three. The first group, 1a and 2a, grows on media supplemented

Table 2. Response pattern of the five mutants

Amino acids (100 μg/ml)	Mutant strains				
	1a	2a	3a	4a	3d
D-Glutamic acid	+[1]	+	+	+	+
D-Aspartic acid	+	+	+	+	+
D-Alanine	+	+	−	−	+
L-Glutamic acid	−	−	+	+	−
L-Aspartic acid	−	−	+	+	−
L-Alanine	+	+	−	−	−
DL-Glutamic acid	+	+	+	+	+
DL-Aspartic acid	+	+	+	+	+
DL-Alanine	+	+	−	−	+
L- or DL-Valine	+	+	−	−	−
L-Proline	−	−	+	+	−
L-Arginine	−	−	+	+	−
MM[2]	−	−	−	−	−
CD[3]	+	+	+	+	−

1) The symbol, +, indicates positive growth.
2) Minimal medium
3) Minimal medium supplemented with 0.1% casein hydrolysate and 100 μg/ml D-glutamic acid.

The amino acids tested in this experiment are as follows: D-, L-, DL-glutamic acid, D-, L-, DL-aspartic acid, D-, L-, DL-alanine, L-glutamine, L-asparagine, L-histidine, L-proline, L-arginine, L-, DL-valine, L-, DL-isoleucine, L-leucine, L-, DL-threonine, L-, DL-methionine, L-cystine, DL-tryptophan, L-tyrosine, L-, DL-serine, glycine and L-lysine.

with either D-glutamic acid, D-aspartic acid, D-alanine, L-alanine, DL-glutamic acid, DL-aspartic acid, DL-alanine, or L-valine; the second group, 3a and 4a, grows on media supplemented with either D-glutamic acid, D-aspartic acid, L-glutamic acid, L-aspartic acid, DL-glutamic acid, DL-aspartic acid, L-proline, or L-arginine; the third group, 3d, grows on media with either D-glutamic acids, D-aspartic acid, D-alanine, DL-glutamic acid, DL-aspartic acid, or DL-alanine. The mutants belonging to the first and the second groups grow on CD medium, while the mutant 3d does not. The reason will be referred to later.

4. *Growth in media containing* D- *or* L-*glutamic acid*

Selecting one representative from each group, the optimum concentrations of D-glutamic acid in DG medium capable of supporting the full growth of these mutants were searched for. Cells were cultivated in liquid media

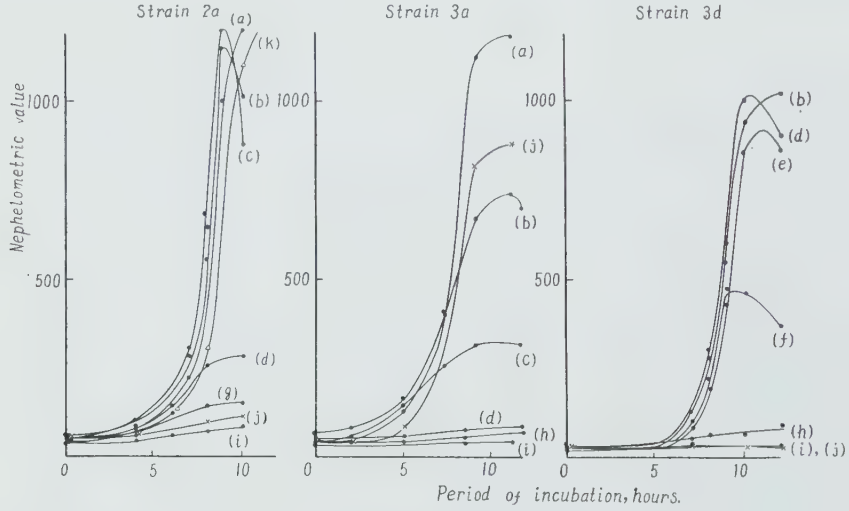

Fig. 1. Growth in media supplemented with various concentrations of glutamic acid.

Concentrations of D-glutamic acid (μg/ml): (a) 200, (b) 100, (c) 50, (d) (10), (e) 5, (f) 3, (g) 2, (h) 1, (i) 0. (j) 100 μg/ml L-glutamic acid, (k) 100 μg/ml DL-glutamic acid.

(the method has been described previously), and growth curves were produced. As seen in Figs. 1 and 2, the optimum concentrations differ from strain to strain. Mutant 3a demands about 200 μg/ml and mutant 2a 50 μg/ml, while mutant 3d demands 5 μg/ml D-glutamic acid for its full growth. Among the three mutants tested, 3a is the only mutant that can grow in the presence of L-glutamic acid as well as of D-glutamic acid.

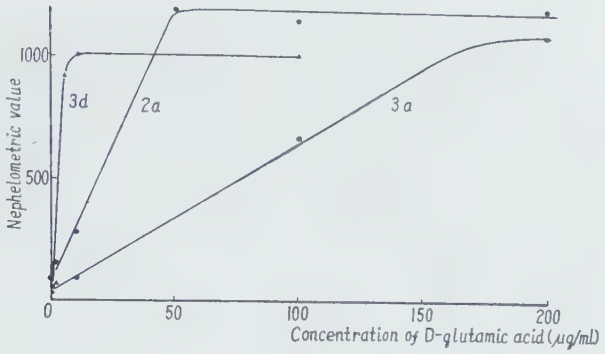

Fig. 2. Dose-growth relationship (translated from Fig. 1).

5. *Inhibition of growth by* L-*amino acids*

As described previously, mutant 3d can not grow in CD medium in spite of the fact that the medium contains D-glutamic acid. It was thought therefore that the negative growth might be due to the mutual effect between two amino acids. Experiment was carried out by the auxanographic method, and the presence or absence of inhibition zone was checked. As a result, the following amino acids showed inhibitory effect against the growth of 3d in the presence of D-glutamic acid, L-glutamic acid, L-aspartic acid, and L-serine. Besides, L-glutamine and L-asparagine showed slight inhibition. It must be noted here, however, that the inhibitory effect of L-serine is not specific. The amino acid inhibits the growth of wild-type strain of *B. subtilis* K too. Hence application of mutant 3d for the assay of D-glutamic acid in natural products is unpromissing.

DISCUSSION

So far, the author has described the mutants as D-glutamic acid-requiring mutants. These mutants can not grow in minimal medium unless D-glutamic acid is supplemented, in which sense they are D-glutamic acid-less mutants. However, mutants 1a and 2a (group 1) grow in a medium in which D-glutamic acid is replaced with L-alanine, and mutants 3a and 4a (group 2) in a medium replaced with L-glutamic acid or L-aspartic acid. Therefore, they are not D-glutamic acid-less mutants in a strict sense. Meanwhile, mutant 3d (group 3) is able to grow only when supplied with D-glutamic acid, D-aspartic acid, or D-alanine. This is the true D-glutamic acid-less mutant.

It is uncertain whether the attempt reported in this paper is the first in the field of microbiology. Nevertheless, the author believes that this kind of experiment must be repeated. D-Amino acid-less mutants would become a useful material for solving difficult problems underlying the study of cell wall, spore, and antibiotic production. The two papers which follow will deal with these problems carried out in this connection.

SUMMARY

To produce D-glutamic acid-requiring mutants, spores of *Bacillus subtilis* K were exposed to X-rays. The mutants were selected by the replica plating method following the penicillin screening. Using a medium enriched with D-glutamate in stead of the so-called complete medium, five D-glutamate dependent mutants were isolated. They were classified into three groups in terms of the growth response pattern (Table 2). Mutant 3d grows in minimal medium when it is supplemented with either D-glutamic acid, D-aspartic acid, or D-alanine. Meanwhile the mutant does not grow in the media when L-aspartic acid or L-glutamic acid coexists.

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STUDIES ON MUTANTS OF *BACILLUS SUBTILIS* REQUIRING D-GLUTAMIC ACID
II. UNBALANCED GROWTH DEATH OBSERVED IN MUTANT 3d

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It is known that unbalanced synthesis of cell wall and cytoplasm brings a bacterium to death (unbalanced growth death). For example, a mutant of *Escherichia coli* incapable of synthesizing diaminopimelic acid will die of itself provided that the acid is not supplied (*1*, *2*). The phenomenon is understood to be an indication that the synthesis of cytoplasm proceeds independently of that of cell wall in which diaminopimelic acid is an essential component, and, owing to the lack of balance, the cells perish. Because D-glutamic acid is known to be a component of cell wall in a certain kind of bacteria, a similar situation is conceivable. To test this possibility, a mutant of *Bacillus subtilis* requiring D-glutamic acid for growth was placed in a medium devoid of D-glutamic acid.

MATERIALS

Strains: A wild-type strain and the following two mutants of *B. subtilis* K were used. 3d is a mutant which is unable to synthesize D-glutamic acid, D-aspartic acid, and D-alanine. This inability is due to the lack of an enzyme, alanine racemase (see the following paper). 3a is a mutant which is incapable of synthesizing both D- and L-glutamic acid.

Media: MM medium and DG medium were described in the preceding paper (*3*). M-N is a modified minimal medium containing no nitrogen sources (ammonium chloride and ammonium nitrate).

METHODS AND RESULTS

1. *Growth of 3d in DG, MM, and M-N media*

Although growth of mutant 3d in DG and MM media had been expressed with nephelometric values, it was attempted in this experiment to produce growth curves by counting viable cells. It was done so because the nephelometric values do not always reflect real viable cell numbers. To count

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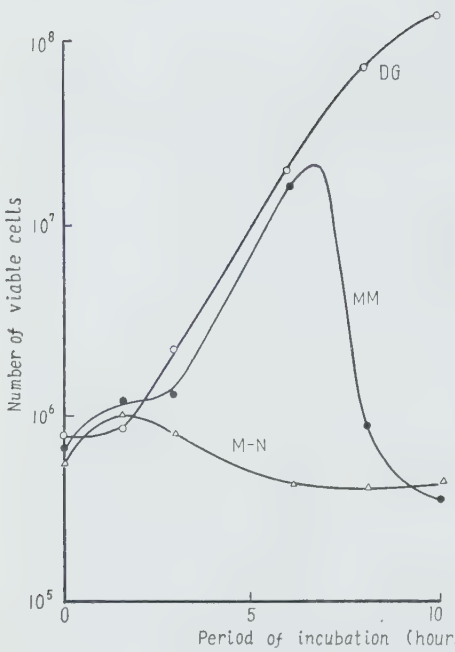

Fig. 1. Growth curves of strain 3d
MM: minimal medium, DG: MM plus 100 μg/ml D glutamic acid, M-N: MM minus nitrogen source.

viable cells, aliquots were withdrawn periodically from the cultures on a seesaw-type shake machine, and plated onto DG agar medium after appropriate dilution. Cultivation was carried out at 37°.

As the curves in Figure 1 indicate, the viable cells in DG medium increased continuously with time lapse, while the cells in MM medium increased for a while and decreased rapidly after a lapse of seven hours. The latter type of growth curve will be referred to as "residual growth curve." It is because growth was determined nephelometrically that no residual growth was observed in Figure 1 of the preceding paper (*3*). When the mutant was cultivated in M–N medium, the viable cells increased but only to a negligible degree.

2. *Growth of 3a in DG and MM media*

Similarly, mutant 3a was cultivated in DG and MM media. In contrast to mutant 3d which showed residual growth in MM medium, the viable cell numbers did not change significantly throughout the whole course of incubation. Results are shown in Figure 2.

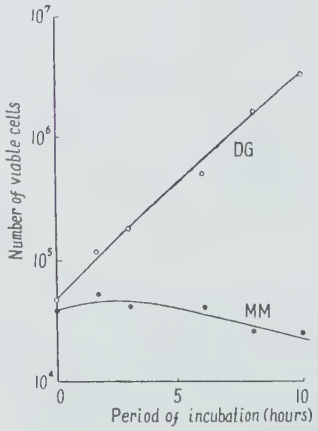

Fig. 2. Growth curves of strain 3a.
MM: minimal medium
DG : MM plus 100 μg/ml D-glutamic acid

3. *Presence of D-glutamic acid in cell wall*

In order to test whether or not D-glutamic acid would be a component of the cell wall of *B. subtilis* K, the following experiments were carried out. Vegetative cells were harvested from a culture of wild-type strain and ground in a mortar with fine glass beads. Residual intact cells were spinned out at 1,000×g for 15 minutes, and cell walls in the supernatant were collected at a higher speed (10,000×g, 15 minutes). The cell walls thus obtained were

washed twice with 1 M NaCl and distilled water. The yield was 0.1 g in dry weight per 16 g wet cells.

50 mg of the dry material in 5 ml of 3N hydrochloric acid was sealed in a small tube, and heated at 110° for 20 hours. The hydrolysate was dried up *in vacuo* and humins that appeared after the addition of water filtered off. This procedure was repeated several times and finally the content was filled up to 1 ml with water. Amino acids in this solution were separated by the chromatographic technique using a mixture of solvents (butanol 4, acetic acid 1, and water 2). Glutamic acid (D or L) was extracted with water from the corresponding area on the paper.

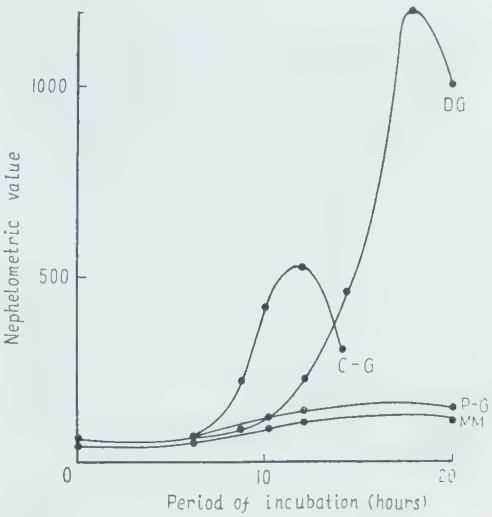

Fig. 3. Growth response of mutant 3d to glutamic acid fraction of cell walls and protoplasts.

MM: minimal medium, DG: MM+10 μg/ml D-glutamic acid, C-G: MM+glutamic acid fraction from cell walls, P-G: MM+glutamic acid fraction from protoplasts (C-G and P-G contain ca. 4 μg/ml glutamic acid respectively.)

L-Glutamic acid was assayed manometrically using glutamic acid decarboxylase prepared from *E. coli* (*4*), while D-glutamic acid was assayed biologically depending on the dosage-growth curve given in Figure 2 of the preceding paper (*3*). Total glutamic acid was assayed by the ninhydrin method (*5*). As a matter of fact, the extract mentioned above did support the growth of 3d, the mutant which is able to grow only in the presence of D-glutamic acid (Figure 3). When assayed biologically, 1.65 mg D-glutamic acid was found in 100 mg dried cell walls, amounting to 81.3% of the total glutamic acid contained in the material (Table 1). The remainder is L-form. Thus, the presence of D-glutamic acid in the cell wall of *B. subtilis* K was evidenced.

Table 1. Ratio of D- and L-glutamic acid to total glutamic acid in protoplasts and cell walls

	Amount of glutamic acid in 100 mg of each fraction (mg)			
	Protoplasts		Cell walls	
Total glutamic acid	2.50		2.03	
D-Glutamic acid*	—	0.3	—	1.65
L-Glutamic acid**	2.44	—	0.29	—

* assayed biologically.

** assayed manometrically (see text).

4. *Absence of* D-*glutamic acid in cytoplasm*

Protoplasts were prepared to estimate the amount of D-glutamic acid in cytoplasm of the bacillus. First, 300 mg washed cells were suspended in 1 ml of the solution consisting of 20% sucrose and 0.05 M phosphate buffer (pH 7.0), and then treated with 300 μg lysozyme for three hours at 37°. The precipitate after centrifugation at 10,000 × g for 5 minutes was washed twice with 0.5 M KCl and dried. 1.5 g dried protoplast was obtained from 14 g wet cells.

The material was hydrolyzed by the method described above, and D- and L-glutamic acid were assayed. The glutamic acid fraction prepared from this material did not support the growth of 3d (Figure 3). As a result of the assay, it was found that the fraction consisted of about 98% L-glutamic acid and 2% D-glutamic acid. Because the content of D-glutamic acid in the material is too small to be considered a component of protoplast (cytoplasm and cytoplasmic membrane), it is supposed that the biological significance of D-glutamic acid in cytoplasm, if any, is negligible when compared with that in the cell wall.

DISCUSSION

As has been expected in the introduction, the cells of mutant 3d perished when they were placed in a medium devoid of D-glutamic acid. The residual growth that lasted for seven hours might be due to the surplus D-glutamic acid brought about by inoculum. At the expense of it, cell walls would have been reproduced for a while. Meanwhile, when the surplus D-glutamic acid was used up, there would be no supply of D-glutamic acid, and the cell wall formation would stop. Even at this time, synthesis of cytoplasm would proceed normally, because the strain was capable of synthesizing L-amino acids from raw materials. Thus, the synthesis of cytoplasm and cell wall would lose balance. The growth curve of mutant 3d in M–N medium supports the above mentioned view (Figure 1). In this medium, the synthesis of cytoplasm as well as that of cell wall would not have taken place because of the lack of nitrogen sources. On account of this balanced deficiency, no significant residual growth resulted. The absence of residual growth in the culture of mutant 3a in MM medium (Figure 2) is considered to be due to the same reason. The mutant can not synthesize both D- and L-glutamic acid.

D-Glutamic acid was found mainly in cell wall fraction and in a negligible amount in cytoplasm fraction. This result is in agreement with the data so far published (*6*). It is concluded, therefore, that the death of mutant 3d in MM medium is a similar phenomenon to that found in a diaminopimelic acid requiring mutant of *E. coli* (*1*, *2*). In the latter case, diaminopimelic acid is known to be a component of cell wall of the bacterium.

SUMMARY

When mutant 3d is incubated in a medium devoid of D-glutamic acid, the number of viable cells increases for a while and after the lapse of seven hours begins to decrease abruptly. Because D-glutamic acid is a major component of cell wall and a minor component of cytoplasm, and because the strain cannot synthesize D-glutamic acid although it can synthesize L-glutamic acid and other L-amino acids, it is concluded that the phenomenon mentioned above is caused by the unbalanced synthesis between cell wall and cytoplasm.

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STUDIES ON MUTANTS OF *BACILLUS SUBTILIS* REQUIRING D-GLUTAMIC ACID
III. ENZYME ACTIVITIES OF THE MUTANTS

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As reported previously (*1*), the five mutants of *Bacillus subtilis* K requiring D-glutamic acid for growth are classified into three groups. Mutants 1a and 2a grow on media supplemented with either L-alanine, D-alanine, D-aspartic acid or D-glutamic acid, mutants 3a and 4a on media with either L-aspartic acid, L-glutamic acid, D-aspartic acid or D-glutamic acid, and mutant 3d on media with either D-alanine, D-aspartic acid or D-glutamic acid. These dependencies were thought to be caused by the lack of enzymes involved in the biosynthesis of these amino acids. It is not unusual in microorganisms that a strain turns out incapable of producing an enzyme as a result of gene mutation (*2*). On this basis, several enzymes that were considered important for the biosynthesis of D- and L-glutamic acid were investigated.

MATERIALS AND METHODS

Strains: A wild-type strain and the four mutants, 2a, 3a, 4a, 3d were employed.

Media: Medium A consists of components of minimal medium (*1*), 0.1% nutrient broth (v/v) and 200 μg/ml of DL-glutamic acid. B is a medium lacking nutrient broth of medium A. Medium A is used for mutants 2a, 3a and 4a, and medium B for 3d.

Preparation of cell extract: Mutants, 2a, 3a and 4a were cultivated in 100 ml of medium A at 30° for 15–17 hours, and mutant 3d in medium B for 17–20 hours. These cultivations were carried out on a shake machine. Cells at the late logarithmic phase were harvested by centrifugation and washed several times with 0.1 M phosphate buffer (pH 7.2). The washed cells were suspended heavily in 0.01 M phosphate buffer (pH 7.2, ca. 50 mg wet cells per ml) and treated by a 10 kc sonic oscillator (Tōyō Rikō, Type 50–2) for 20 minutes. The resulting disrupted cells were centrifuged at 10,000 $\times$g for 15 minutes, and the supernatant thus obtained was dialysed against 0.01 M phosphate buffer (pH 7.4) for 20 hours at 5°. This preparation is to be called "dialysed extract."

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Measurement of enzyme activities (1) Racemases — To measure the activities of enzymes (racemases) that catalyse the reaction between L-alanine and D-alanine (*3*) and between L-glutamic acid and D-glutamic acid (*4*, *5*), 0.4 ml dialysed extract, 40 μg pyridoxal phosphate and 100 μmole L-alanine or D-glutamic acid were dissolved in 1 ml of 0.1 M phosphate buffer (pH 8.0). The mixture was incubated at 37° for 40 minutes, and D-alanine and L-glutamic acid formed were measured by the method to be described in a later section. The specific activity is defined as "amounts of D-alanine or L-glutamic acid formed (μmole)/mg protein/hour."

(2) Transaminases (*6*)—Transaminase activities were measured as follows. 0.4 ml dialysed extract, 40 μg pyridoxal phosphate, and each 100 μmole D-glutamic acid and pyruvic acid or each 100 μmole L-alanine and α-ketoglutaric acid were mixed in 1 ml of 0.1 M phosphate buffer (pH 8.0), and D-alanine or L-glutamic acid formed after 40 minutes at 37° was measured. The specific activity is defined as "amounts of D-alanine or L-glutamic acid formed (μmole)/mg protein/hour."

(3) Dehydrogenases (*7–9*)—To measure the activities of L-alanine-, L-glutamic acid- or L-aspartic acid-dehydrogenases, a reaction mixture consisting of 0.4 ml dialysed extract, 10 μmole L-alanine (or L-glutamic acid, or L-aspartic acid), 0.5 μmole DPN (or TPN) dissolved in 3 ml sodium pyrophosphate buffer (pH 8.3) was prepared. The mixture was placed at room temperature (23°–25°), and optical density was measured at intervals using a Shimazu spectrophotometer. Reaction after the addition of each substrate was recorded. The specific activity is defined as "change in absorption values at 340 mμ/mg protein/minute."

Determination of L-glutamic acid and D-alanine: To stop reactions, trichloroacetic acid (TCA) was added in a concentration of 5% (w/v). The mixture was centrifuged, and then the supernatant was treated with ether to remove the TCA. L-Glutamic acid or D-alanine in the sample was assayed manometrically by using L-glutamic acid decarboxylase prepared from *Escherichia coli* for the determination of L-glutamic acid (*10*), and D-amino acid oxidase prepared from sheep kidney for D-alanine (*11*). Protein was determined by the method of Lowry et al (*12*) using bovine serum albumin as the standard.

Chemicals: D-, L- and DL-amino acids used in this work were supplied from Ajinomoto Co. Inc., DPN and TPN from Sigma Chemical Co., and pyridoxal phosphate, pyruvic acid and α-ketoglutaric acid from Nutritional Biochemical Cooperation.

RESULTS

Alanine and glutamic acid racemase activities

It is considered that racemases are essential catalizers for the synthesis of D-amino acids (*6*). To know whether the wild-type strain and the mutants might possess the racemases or not, their cell free extracts were reacted

with D-(or L-) alanine or D-(or L-) glutamic acid in the presence of pyridoxal phosphate. The result was that the crude extract from the wild-type strain could convert D-alanine to L-alanine and *vice versa*. Meanwhile, no conversion of D-(L-)glutamic acid to L-(D-)glutamic acid was brought about by the extract (figure 1). Moreover, the latter reaction was not found even when the extract was prepared from the cells at different growth phases or under conditions where reaction mixtures of different pH values were employed. Therefore, it is concluded that alanine racemase plays an important role in the biosynthesis of D-amino acids, but glutamic acid racemase does not.

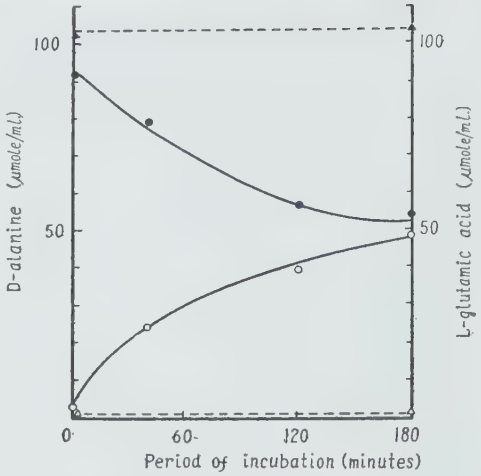

Fig. 1. Alanine and glutamic acid racemase activities in a wild-type strain.

The reaction mixture consists of 1 ml undialysed extract, 500 μmole alanine or glutamic acid (D or L), 100 μg pyridoxal phosphate and 0.02 M phosphate buffer (pH 8.0). The final volume is 5 ml. Substrates: ● D-alanine, ○ L-alanine, △ D-glutamic acid, ▲ L-glutamic acid.

Table 1 shows racemase activities of the wild-type strain and the four mutants. Although glutamic acid racemase activity was not found in the mutants as in the case of wild-type strain, alanine racemase activities were detected in all the strains tested. Among them, the activities in mutants 3a and 4a were higher than in the wild-type, and the activity in mutant 3d was equivalent to 10% of that in the wild-type. Since the addition of crude extract of mutant 3d to the extract of the wild-type did not reduce the enzyme activity, it is assumed that the low alanine racemase activity in mutant

Table 1. Alanine and glutamic acid racemase activities.

Strains	Specific activity*	
	Alanine racemase	Glutamic acid racemase
2a	72.0	0
3a	92.5	0
4a	149.5	0
3d	4.9	0
Wild strain K	55.0	0
K+3d**	60.1	—

* See text.

** K+3d indicates a mixture of dialysed extract of K and crude extract of 3d (1:1).

3d is not due to the co-existence of inhibitory substances. This supposition is supported by fact that the mutant 3d regains the normal alanine racemase activity when it reverts to prototrophs in spontaneous mutation. A similar phenomenon was observed, as shown in Table 2, even in revertants of mutants 2a, 3a and 4a.

Table 2. Alanine and glutamic acid racemase activities in revertants.

Strains**	Specific activity*	
	Alanine racemase	Glutamic acid racemase
2a-R4	60.2	0
3a-R1	71.5	0
4a-R1	50.3	0
3d-R119	66.0	0
Wild strain K	55.0	0

* See text.

** 2a-R4 is a revertant of 2a which was isolated from a plate containing minimal medium and 100 μg/ml L-glutamic acid. 3a-R1 and 4a-R1 were isolated from a plate containing minimal medium and 100 μg/ml DL-alanine, and 3d-R119 from a plate containing complete medium.

Alanine-glutamic acid transaminase activities

The results obtained in the preceding experiments suggest that the negative growth of mutant 3d on L-amino acids and the possitive response on D-amino acids might be due to the low activity of alanine racemase. But, since it was not the case with other mutants, an attempt was made to examine their transaminase activities (Table 3). As a result of experiments aiming at measuring the transamination between glutamic acid and alanine, it was found that mutant 4a possessed a low activity of transaminase, about 25% of that in the wild-type strain, and mutant 3a, which is similar to 4a in its response pattern, possessed an activity almost equal to that of the wild-type. It was also shown that L-alanine-L-glutamic acid transaminase activity in these strains is, in general, lower than D-alanine-D-glutamic acid trans-

Table 3. Alanine-glutamic acid transaminase activities.

Strains	Specific activity*	
	D-ala-D-glu	L-ala-L-glu
2a	21.4	1.17
3a	29.9	4.55
4a	4.87	4.72
3d	23.8	0.35
Wild strain K	19.7	2.31

* See text.

aminase activity of the same strains. The difference was greatest in mutant 3d.

Alanine-, Glutamic acid-, Aspartic acid-dehydrogenase activities

The dehydrogenases were studied by using DPN, TPN, or a mixture of them as the hydrogen accepter. As the data in Table 4 show, L-alanine-

Table 4. Alanine-, glutamic acid- and aspartic acid-dehydrogenase activities*.

Strains	Specific activity** ($10^3 \Delta E_{340}$/min/mg protein)		
	AlDH (DPN)	GlDH (DPN, TPN)	AsDH (DPN, TPN)
2a	13.2	0	0
3a	29.0	0	0
4a	35.0	0	0
3d	42 3	0	0
Wild strain K	104	0	0

* The abbreviations used are: AlDH, L-alanine-dehydrogenase; GlDH, L-glutamic acid-dehydrogenase; AsDH, L-aspartic acid-dehydrogenase.

** See text.

dehydrogenase was found in all the mutants tested as well as in the wild-type strain, while L-aspartic acid- and L-glutamic acid-dehydrogenases were not. The results coinside with the data reported by BRAUNSTEIN (*13*). Although alanine-dehydrogenase activities of the mutants were, generally speaking, lower than that in the wild-type, the activity in mutant 2a was exceptionally low. This finding supports the following view though not conclusively. If D-amino acids are produced via L-alanine as it is widely believed, a defect in alanine-dehydrogenase production would disturb the synthesis of these amino acids, and as a result, the requirement of L-alanine, D-alanine, D-aspartic acid, and D-glutamic acid for growth would appear. Because mutant 2a really depends on these amino acids, this hypothesis would be allowed only when the synthesis of L-aspartic acid and L-glutamic acid from any sources other than L-alanine is evidenced. To test this possibility, products from fumaric acid formed by the extract of mutant 2a was examined by paper chromatogram, and, as it was expected, the presence of two L-amino acids was ascertained.

DISCUSSION

It is by no means easy to determine the correlation between growth velocity and enzyme activity. A strain of low enzyme activity does not always grow slowly unless the activity is below the limiting degree, and what is important, the limiting degree is different from strain to strain. The second difficulty is that the enzyme activity determined with cell free extract does not represent the actual activity in the intact cell. Unfortunately, these

problems have not yet been solved completely in microbiology notwithstanding its importance in dealing with experimental results.

Nevertheless, it is allowed in the field of biochemical genetics to discuss the gene-enzyme relationship by assaying enzyme activity of a mutant. In general, a mutant having a defect in a certain gene locus can not produce an enzyme or can it produce an enzyme with low activity (*14*). It may be fair, therefore, to suppose that the D-glutamic acid-requiring mutants reported in the previous paper (*1*) might have a defect in their enzyme system. As seen below, the results in this paper support the view although it is still uncertain whether the defect (the low activity) is below the limiting activity that allows the growth.

Among the four mutants studied, the defect in mutant 3d is most obvious. In the mutant, the alanine racemase activity was about 10% of that in the wild-type strain. If D-amino acids are to be produced via L-alanine and D-alanine as it is widely accepted, the mutant should require D-alanine, D-aspartic acid, and D-glutamic acid. This expectation agrees with the response pattern of mutant 3d. It appears that D-alanine is converted to D-glutamic acid by transamination, and the reaction proceeds towards D-glutamic acid in-living cells, because the amino acid is essential for the formation of cell wall (*15*). It is assumed too that D-aspartic acid is converted to D-glutamic acid under a similar mechanism.

As described in the last part of the preceding chapter, the site of the genetic block in mutant 2a seems to be between pyruvate and L-alanine. The requirement of L-alanine, D-alanine, D-aspartic acid and D-glutamic acid is the result of this genetic block. It is supposed too that the requirement of D-aspartic acid and D-glutamic acid by mutant 4a is caused by the low activity of D-alanine-D-glutamic acid transaminase. But, as a matter fact, the strain requires L-aspartic acid and L-glutamic acid in addition to the D-amino acids. It is necessary therefore to explain this growth reponse pattern to prove simultaneous occurrence of depression and induction, that is, the pathway for the synthesis of L-glutamic acid and L-aspartic acid must be closed and the pathway for the synthesis of D-glutamic acid from L-glutamic acid or L-aspartic acid must be opened. These reactions were examined carefully, but all attempts ended in failure.

As mutant 3a, a spontaneous mutant of 3d, requires L-glutamic acid, L-aspartic acid, D-glutamic acid and D-aspartic acid just like mutant 4a in spite of its higher activity of D-alanine-D-glutamic acid transaminase, the correlation between enzyme activity and requirement is not so simple as to allow us to draw a ready conclusion. The authors' favorite opinion is that these enzymes are related to each other in their formation, and the requirement is the combined effects of genetic damage and the interaction between two or more enzyme forming systems. BRAUNSTEIN's finding (*13*) that a Am mutant of *B. subtilis* turned out to be a producer of L-glutamic acid-dehydrogenase in compensation of L-alanine-dehydrogenase is very interesting in this respect.

SUMMARY

To look for the site of genetic block in the mutants of *Bacillus subtilis* K requring D-glutamic acid, several enzymes that were considered important for the biosynthesis of D- and L-glutamic acid were investigated. In mutant 3d, alanine racemase activity was about one tenth of that in the wild-type strain. It appears therefore that the block site in this mutant is between L-alanine and D-alanine. The mutant requires D-alanine, D-aspartic acid, or D-glutamic acid for growth.

L-Alanine dehydrogenase activity was exceptionally low in mutant 2a. Because the mutant is able to synthesize L-aspartic acid and L-glutamic acid from fumarate too, the requirement of L-alanine, D-alanine, D-aspartic acid, or D-glutamic acid may be due to the slow reaction between pyruvate and L-alanine.

Mutant 4a possesses a low activity of D-alanine-D-glutamic acid transaminase and responds to L-aspartic acid, L-glutamic acid, D-aspartic acid, or D-glutamic acid. The dependency on D-aspartic acid or D-glutamic acid is reasonable if these D-amino acids are produced from D-alanine by transamination, while the dependency on L-aspartic acid or L-glutamic acid remains unexplained. The correlation between a genetic damage and an enzyme formation was discussed in this connection.

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